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Methods for investigating the bacteria in young Cheddar cheese: inhibition of starter streptococci with bacteriophage and α-bromopropionic acid

By P. S. ROBERTSON*

The Dairy Research Institute (N.Z.), Palmerston North, New Zealand

(*Received* 15 *May* 1959)

SUMMARY. The investigation of the non-starter bacterial flora of Cheddar cheese during the first few weeks of the ripening period is rendered difficult by the great preponderance of starter streptococci in the bacterial population. It was found that the starter streptococci could be eliminated from agar plates containing various dilutions of the emulsified cheese by the use of phage specific for the pure culture starters used to make the cheese or alternatively by the use of α-bromopropionic acid.

The successful use of the phage method of eliminating lactic streptococci from bacterial mixtures in poured agar plates depends on the use of single strain starters, and on the absence of phage-resistant forms in the culture used. The latter objective may be achieved by use of starter strains showing a low proportion of resistant cells in a normal culture and by taking precautions to avoid excessive phage development during the cheesemaking process.

In the first few weeks of the ripening period the predominance in Cheddar cheese of lactic streptococci from the starter makes it very difficult to obtain an accurate picture of the rest of the bacterial flora. Yet a knowledge of the incidence of bacterial strains and species apart from those derived from the starter is desirable because they may play an important part in the development of cheese flavour. They may act by means of extra- or intracellular enzymes even if they survive for only a short time in the ripening cheese. A short-lived species in the cheese may be completely concealed and thus escape isolation.

The development of selective media (e.g. the acetate medium of Mabbitt & Zielinska, 1956) has made it possible to determine the numbers of specific types of bacteria (e.g. lactobacilli) in young cheese, but their significance in the total non-starter flora has still remained obscure.

In this paper two methods are described which seem to enable all the non-starter bacteria to be counted and isolated from cheese during and immediately after its manufacture. The two methods depend upon:

(*a*) The use of α-bromopropionic acid in poured agar plates for inhibiting the lactic streptococci. This acid was used by Benchetrit (1933) for the suppression of lactic streptococci in agar plates containing dilutions of butter starter.

* At present at the National Institute for Research in Dairying, Shinfield, Reading.

(*b*) The use of a specific bacteriophage in poured agar plates to lyse the starter streptococci.

Both methods are very effective, provided certain conditions are satisfied.

MATERIALS AND METHODS

Five Cheddar cheeses were used to investigate the usefulness of the two proposed methods for estimating the numbers of non-starter bacteria. Three 80 lb cheeses were made from high-quality flash pasteurized milk at The Dairy Research Institute (New Zealand). The starters used were pairs of single strains of *Streptococcus lactis* and *Str. cremoris*, namely, cheese A, ML_1 and ML_3; cheese B, ML_4 and US_3; Cheese C, C_{13} and E_8. The first two pairs of strains had not been used in the factory for a period of 5 months before the present experiments, while the last pair had been used for cheesemaking about twice each week for the preceding 7 months. As was to be expected, there were large numbers of phage particles in the last cheese (6×10^8/g) and relatively few in the first two cheeses (A, 1×10^5/g; B, 3×10^6/g). Two 40 lb cheeses were made from high-quality milk at the National Institute for Research in Dairying, England. One of these cheeses was made from raw and the other from pasteurized milk (160 °F for 17 sec). Two single strain cultures (509 and 924) were used in the manufacture of the first cheese (D) and culture 804 alone was used as a starter for the second cheese (E). Extensive phage development in the whey was not expected in the manufacture of either of these cheeses since none of the starter strains selected had been used in the factory on more than five occasions in the preceding 3 months.

The New Zealand cheeses were sampled by inserting a sterile butter-trier into the upper flat surface of the cheese and withdrawing a plug about two-thirds the height of the cheese in length. The lower half of this plug (30–40 g of cheese) was weighed into a blender jar containing 100 g of sterile distilled water. A similar weight of cheese was obtained from the English cheese by withdrawing three plugs with a cheese-trier. The sample holes in the cheeses were rammed full with grated cheese of similar age and the tops sealed with molten wax. Subsequent samples were drawn at the same radius from the centre (4½ in.) but at least 1½ in. from the site of the previous sample.

Cheese samples were blended (Ato-mix blender) for ½ min at slow speed, then for 2 min at full speed. To allow the foam to subside, but to avoid excessive separation of the cheesefat, the emulsion was allowed to stand for exactly 2 min before dilutions were prepared.

Media. Except where otherwise stated, a lactose—yeast—phosphate agar (LYPA) of the following composition was used: lactose 2%, Oxoid peptone 0·8%, Oxoid beef extract 1·0%, Oxoid yeast extract 0·2%, disodium hydrogen phosphate (anhydrous) 0·25%, Davis agar (N.Z.) 1·0%. This is a modification of the medium of Hunter (1946). The corresponding broth (LYPB) contained the same ingredients, except the agar, but twice the concentration of phosphate.

The selective media acetate agar (AcA) and acetate broth (AcB) were made according to the methods of Mabbitt & Zielinska (1956).

Phage method. Preliminary trials were made to find the concentration of phage

that would completely inhibit the phage-susceptible cells in plates prepared with dilutions of 24 h milk cultures of various single strain starters. It was found that 0·25 ml of a high-titre bacteriophage preparation (at least 10^7 particles/ml, prepared by the method of Whitehead & Bush, 1957) was as effective as 10 times this volume in inhibiting the phage-susceptible cells of the strains tested.

When the non-starter flora of a cheese was to be examined by the phage method, 0·25 ml of neat phage preparation was added to 1 ml of a dilution of cheese emulsion at least 10 min before the addition of LYPA at 45 °C.

Table 1. *Effect of various concentrations of α-bromopropionic acid in LYPA on the growth of dairy bacteria in poured and on streaked plates*

			Concentration of α-bromopropionic acid (M × 10^4)						
Species	Strain	Source*	0	1	2	4	12	24	40
Str. cremoris	ML_1	a	++	++	++	−	.	.	.
	ML_4	a	++++	++++	+++	−	.	.	.
	US_2	a	+++	+++	++	−	.	.	.
	HP†	a	++	++	++	−	.	.	.
	924	b	+++	+++	++	−	−	−	−
	804	b	+++	+++	+++	++	+	−	−
Str. lactis	ML_3	a	++++	++++	++++	++++	++++	+++	−
	C_{10}	a	++++	++++	++++	++++	++	−	−
	509	b	++++	++++	++++	++++	++++	++	−
L. plantarum	10·2	c	++++	.	.	++++	++++	++	−
	10·5	c	++++	.	.	++++	++	+	−
	21·1†	c	.	.	+++	+++	++	.	−
L. casei	25·2†	c	.	.	+++	+++	++	.	−
L. casei-plantarum	19·1†	c	.	.	+++	+++	++	.	−
L. casei var. *rhamnosus*	7469†	d	.	.	+++	+++	++	.	−
L. brevis	1·2	c	++++	.	.	++++	++++	++++	++++
Leuconostoc sp.	29·2	c	++++	.	.	++++	++++	+++	++
Micrococcus sp.	L_1†	e	.	.	++++	++++	++	.	−
	L_9	f	++++	.	.	++++	+	−	−
Pediococcus sp.	M_2†	f	.	.	++	++	++	.	−
	A_2†	g	.	.	+++	+++	++	.	−
	8042†	h	.	.	+++	+++	++	.	−
Pseudomonas putrefaciens	Ps26	i	++++	.	.	++++	−	−	−

Magnitude of growth: − = no growth; ++++ = heavy growth.

* a, DRI (N.Z.) cheese-starter collection; b, NCDO, England; c, N.Z. Cheddar cheese (Sherwood, 1939); d, ATCC number (see Sharpe, 1955); e, raw milk (Robertson); f, N.Z. Cheddar cheese (Robertson); g, N.Z. Cheddar cheese (Dacre, 1958); h, ATCC number (Garvie, 1959); i, butter (from Dr P. R. Elliker, U.S.A.).

† Measured on streaked plates only.

α-Bromopropionic acid method. Tests were made of the relative α-bromopropionic acid tolerance of six strains of *Str. cremoris*, three strains of *Str. lactis* and fourteen other species of importance in dairy products. These trials were usually made with poured and streaked plates of LYPA containing various concentrations of redistilled α-bromopropionic acid from 4×10^{-5} to 4×10^{-3}M. A concentration of 4×10^{-4}M was chosen where starter streptococci were to be inhibited in dilutions of cheese emulsion, for the preliminary trials had shown that several strains of *Str. cremoris* were inhibited at this level of acid while all other species tested were unaffected (Table 1). The

inhibitory action of α-bromopropionic acid is dependent on phosphate concentration (Genevois & Nicolaieff, 1932) which must be closely controlled.

Counting and identification of colonies. Duplicate poured plates from appropriate dilutions of cheese emulsion were counted after being incubated aerobically at 30 °C for 5–7 days. Twenty adjacent colonies were picked from one area of an uncrowded plate into yeast milk (YM) and incubated at 30 °C. The results of further examination of these cultures is beyond the scope of the present paper, except in so far as the presence or absence of streptococci from the starters is concerned. Cultures which coagulated the YM in less than 30 h proved on microscopic examination without exception to be streptococci. They were similar in morphology to one or other of the starter strains used, and in most instances it was possible to confirm the identification by phage typing. None of the cultures which took over 30 h to coagulate in YM was a lactic streptococcus.

RESULTS

Cheeses A and B

Changes in the total and non-starter counts of these two cheeses were followed from the time of manufacture until the starter streptococci ceased to be dominant. Samples were plated in LYPA, LYPA plus phages capable of lysing both the starter strains, LYPA plus α-bromopropionic acid and usually in AcA. Detailed results (Fig. 1) are presented only for cheese B, for the results with cheese A were similar

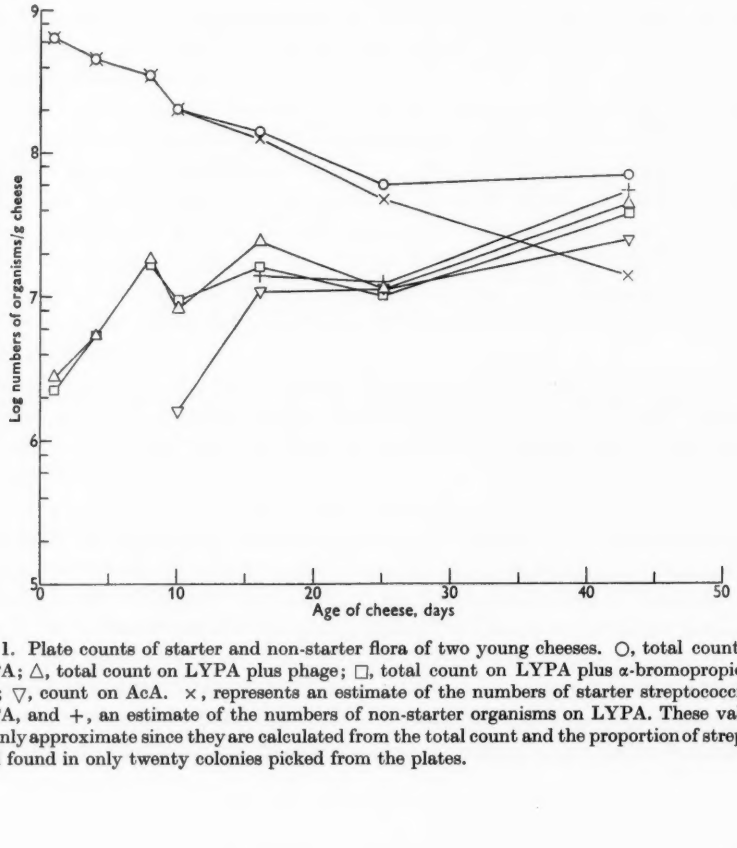

Fig. 1. Plate counts of starter and non-starter flora of two young cheeses. ○, total count on LYPA; △, total count on LYPA plus phage; □, total count on LYPA plus α-bromopropionic acid; ▽, count on AcA. ×, represents an estimate of the numbers of starter streptococci on LYPA, and +, an estimate of the numbers of non-starter organisms on LYPA. These values are only approximate since they are calculated from the total count and the proportion of streptococci found in only twenty colonies picked from the plates.

but less clear-cut since ML_3, a strain of *Str. lactis*, was not completely inhibited by the α-bromopropionic acid. Starter ML_3 was the only lactis strain among the six used in the New Zealand-made cheeses.

Cheese B was examined on seven occasions during the 6 weeks following manufacture. The only streptococci isolated were from the plain LYPA plates; they were identified as strain ML_4 by phage typing. No cultures of strain US_3 were isolated. For the first four examinations all the twenty colonies picked from LYPA were starter streptococci; the first non-starter bacteria were isolated from the 16-day-old cheese and from then on the proportion of streptococci found in the twenty cultures derived from the LYPA plates indicated that the numbers of streptococci in the cheese continued to decrease logarithmically. Only one-quarter of the isolates from the 43-day-old cheese were streptococci. The total count on LYPA declined steadily for the first 25 days. This decline had been arrested by the 43rd day, by which time the non-starter bacteria were dominant.

The counts on the two starter-inhibiting media (phage and α-bromopropionic acid) increased rapidly during the first few days after manufacture, but thereafter the increase was more gradual. No streptococci were isolated from any of these plates and this, combined with the fact that the counts on the two media were closely correlated, is evidence of the success of the methods in eliminating streptococci and disclosing on the plates the non-starter bacteria.

Counts on AcA which specifically favours lactobacilli and some pediococci (Mabbitt & Zielinska, 1956) were much lower than the total non-starter count until the cheese was 16 days old. The cultures isolated from the plates were all lactobacilli or pediococci.

Limitations of the phage method

Cheese C. Extensive phage development during cheesemaking

The results obtained with this cheese illustrated the limitations of the phage method. Although the starter cultures C_{13} and E_8 had behaved normally in the cheese vat and the resultant cheese was of good quality, there had been considerable phage development during the manufacturing process (6×10^8 phage particles/g cheese), though not sufficient to lyse the starter before the required acidity had been produced. Counts from the young cheese on LYPA containing the two phages were almost as high as the counts on plain LYPA and the majority of the colonies isolated from both media were phage-resistant streptococci morphologically similar to C_{13}. In contrast to this the counts on LYPA plus α-bromopropionic acid were substantially lower and only non-starter organisms were isolated. These results, combined with the knowledge that in pure cultures of C_{13} and E_8 there are normally at least 10,000 more phage-susceptible than phage-resistant organisms, suggest that the phage development in the cheese vat had led to the preferential development of phage-resistant cells after susceptible cells were lysed during (and after) cheese manufacture.

Cheese D. Low bacterial count milk and unsuitable starter strains

The first cheese manufactured at the N.I.R.D. gave unexpected results when an attempt was made to suppress the starter streptococci with phage. Although the resultant count was only $1{\cdot}6 \times 10^{-4}$ of the count on plain LYPA, the only strains

which were isolated from this 15-day-old cheese were phage-resistant streptococci morphologically identical with the starter strain 509. There were less than 3×10^3 organisms other than starter streptococci/g of this cheese. A subsequent examination of strain 509 showed that it had an abnormally high proportion of phage-resistant cells. In this instance the very low numbers of non-starter bacteria, combined with an abnormally high proportion of phage-resistant cells in the starter culture, resulted in a situation where the addition of phage did not allow the non-starter flora to be revealed.

α-Bromopropionic acid did not eliminate the starter flora of cheese D. This was expected, for it had been observed (Table 1) that 4×10^{-4}M-α-bromopropionic acid does not inhibit the *Str. lactis* strain 509 which was dominant in this cheese.

Cheese E. Low bacterial count milk and a suitable starter strain

A further cheese was made with low bacterial count N.I.R.D. milk and starter strain 804. This strain was selected because of the low proportion of phage-resistant organisms it exhibited in the pure cultures examined. In this cheese no phage-resistant starter streptococci were isolated from plates containing phage and the non-starter flora of this cheese was revealed. In this second N.I.R.D. cheese, too, the non-starter count was much lower than that of the New Zealand cheeses at the same age (15 days).

DISCUSSION

The use of phage or α-bromopropionic acid to suppress the starter streptococci can be expected to facilitate the investigation of the numerically important strains of non-starter bacteria. Information about the non-starter bacteria which are not sufficiently numerically important to be detected by isolating colonies from starter-free plates can only be obtained by the use of appropriate selective media.

As has already been mentioned, the close correspondence in counts on media containing phage and α-bromopropionic acid indicates that both agents eliminate only the starter bacteria. It is, of course, possible that both the agents used might have inhibited some of the important non-starter bacteria as well as streptococci, but this seems unlikely when the completely different mechanisms of eliminating streptococci (lysis and chemical inhibition) are considered. If some minor strains of bacteria were inhibited by either agent there would be no marked influence on the total count though such an effect might be detected by isolating sufficient colonies from the plates.

Close agreement between the counts on the two media containing inhibiting agents should not be expected with every cheese even if the starter strains used are susceptible to both agents. Apart from phage-resistant streptococci which may develop from a starter strain it is also possible that non-starter streptococci resistant to phage but not to α-bromopropionic acid may occur in some cheeses.

When phage is to be used to suppress the starter streptococci on agar plates the strains for use in the vat must be carefully selected. It is, of course, impossible for this purpose to use a mixed starter culture of unknown composition. Single strains of *Str. cremoris* or *Str. lactis* for which the appropriate specific phages are available are essential. The strains selected must also have a very low proportion of phage-

resistant cells in normal milk cultures. It is possible to reduce the proportion of phage-resistant cocci in a bulk starter culture by use of an inoculum which is sufficiently small to be unlikely to transfer phage-resistant cells from the mother culture.

When phage is to be used it is also advisable to choose a starter culture for which there is little phage in the cheesemaking room either on the equipment or likely to reach the vat as an air-borne infection, otherwise there will be appreciable multiplication of phage in the starter during cheese manufacture. Although this may be too slight to affect acid production it will lead to preferential development of phage-resistant streptococci during cheese manufacture, which may appear on the plates of LYPA plus phage. Large numbers of phage particles in the cheese may lead to lysis of streptococci in the plates themselves, thereby lowering the total count.

When α-bromopropionic acid is used the starters selected should be strains of *Str. cremoris* since some strains of *Str. lactis* are not inhibited by the lower concentrations of the brominated acid which it is necessary to use if inhibition of all non-starter organisms is to be avoided. This inhibiting agent has the advantage that it inhibits phage-sensitive and phage-resistant streptococci without distinction, but the specificity of the phage method has advantages which are attractive and useful provided only that a careful selection of starter cultures is possible.

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The colloidal phosphate of milk

II. Influence of citrate

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Summary. A new method for determining the composition of the colloidal phosphate of milk has been developed, based on analysis of milk free from colloidal phosphate. Preparation of this material is described.

The results suggest that the so-called colloidal calcium phosphate of milk should be more properly described as a colloidal phosphate—citrate.

This colloidal phosphate—citrate shows considerable analytical resemblance to the precipitate formed on neutralizing acidified milk serum. Both can be represented approximately by the empirical formula of a hypothetical citrate apatite.

Uncertainty about the composition of the colloidal phosphate of milk and the nature of its association with caseinate has scarcely diminished since the subject was reviewed some 12 years ago by Horst (1947). Since then the average [Ca]/[P] ratio of the phosphate has been variously reported as 1·44 (Pyne & Ryan, 1950) and 1·35 (White & Davies, 1958) when determined by oxalate titration, as about 1·50 calculated from centrifugal data (Ford, Ramsdell & Landsman, 1955; Edmonson & Tarassuk, 1956), and as about 1·18 when arrived at from analysis of rennet curd (Verma & Sommer, 1957). More recently a [Ca+Mg]/[P] ratio of 1·60 has been proposed largely on analogical considerations (Evenhuis & de Vries, 1957).

A new approach appeared to be needed in view of these conflicting results. Colloidal phosphate-free milk seemed to offer possibilities here; it is relatively easy to prepare and should presumably differ from the parent milk only in its content of colloidal phosphate. Analytical comparison of the two ought therefore to provide an unexceptionable method of arriving at the composition of the phosphate, free from the assumptions in regard to the composition and behaviour of other milk constituents which have so often complicated investigational methods in the past. These expectations have in the event been largely fulfilled and the results have enabled a composition for the colloidal phosphate of milk to be proposed which, it is thought, is more solidly based than most of the compositions previously suggested. A feature of the composition, as will appear, is the inclusion of citrate, so that the colloid now becomes in effect a calcium phosphate—calcium citrate combination. Previous neglect of this citrate component lies at the base of some of the divergent views noted.

As to the nature of the association between colloidal phosphate and caseinate the

position is less clear. Recent opinion tends to favour a relation of simple colloidal protection here. Thus colloidal phosphate has been described as a mere competitor with casein for calcium (von Hippel & Waugh, 1955). Again, the value of the evidence of the formaldehyde titration of milk for casein—phosphate combination has been contested (Evenhuis & de Vries, 1956*a*). Nevertheless, certain aspects of colloidal phosphate behaviour are not easily reconcilable with the current views. This side of the colloidal phosphate problem will be dealt with on another occasion.

GENERAL METHOD OF INVESTIGATION

Essentially the work has consisted of a comparison of the composition of skim-milk with that of a colloidal phosphate-free milk so prepared as to be approximately identical therewith in all respects other than colloidal phosphate content. Direct comparison of the two should, it was anticipated, enable the composition of the colloidal phosphate to be uniquely determined. Oxalate titrations of the two might furnish corroborative evidence.

The colloidal phosphate of milk is normally in equilibrium with a serum approximately saturated in regard to calcium phosphate (Tessier & Rose, 1958), or even slightly supersaturated (Lampitt, Bushill & Filmer, 1937). The level of colloidal phosphate in milk, whether it be that originally present or not, tends therefore to remain unaltered in amount when the milk is dialysed against a normal milk serum. Thus colloidal phosphate contents varying from 0 to 200 % of that of the original milk (which can be attained, for instance, by appropriate acidification or alkalization respectively), have been found to remain unaltered on dialysis against normal milk.

EXPERIMENTAL

Preparation of colloidal phosphate-free milk. Of many possible methods of preparation the most satisfactory—based on the above considerations and already briefly referred to in a previous paper (Pyne, 1958)—proved to be controlled acidification of chilled milk to pH 4·8–5·0, followed by prolonged dialysis against considerable excess of the original milk. This degree of acidification brings the colloidal phosphate completely into solution without causing simultaneous precipitation of casein, and the subsequent dialysis restores to the acidified milk its original composition in other respects. Colloidal phosphate, thus removed, shows no tendency to build up again during this dialysis.

Fig. 1 illustrates the dependence of the degree of removal of colloidal phosphate on the pH attained during acidification. Removal is complete by pH 4·9, as indicated by the attainment at this pH value of the minimum phosphate content for the milk. This content approximates, incidentally, to that of the inorganic phosphate of the corresponding rennet whey.

In practice colloidal phosphate-free milk is prepared by bringing skim-milk at 0 °C to pH 4·9 by addition, with mechanical stirring, of some 0·35–0·40 % by volume of concentrated HCl. Stirring is continued for about 1 h after addition of acid, following which the acidified milk is dialysed for 96 h at 0–5 °C against 24 h changes of some 20 volumes of the original skim-milk. By this time the pH values

of the two milks are found to differ by only about 0·02, and equilibrium between them is taken to be established.

Analytical methods. Inorganic phosphate and calcium were determined by the methods already used (Pyne, 1940). Calcium plus magnesium was determined by EDTA titration (van der Have, 1954), citrate by the method of Saffron & Denstedt as modified by Reinart & Nesbitt (1957) and Marier & Boulet (1958), pH by glass electrode, and oxalate titration by the method of Pyne & Ryan (1950). Other estimations are described as they occur. All recorded analytical values have been corrected for any incidental dilution.

Fresh morning mixed milk from the College Dairy Shorthorn herd was used throughout after removal of fat.

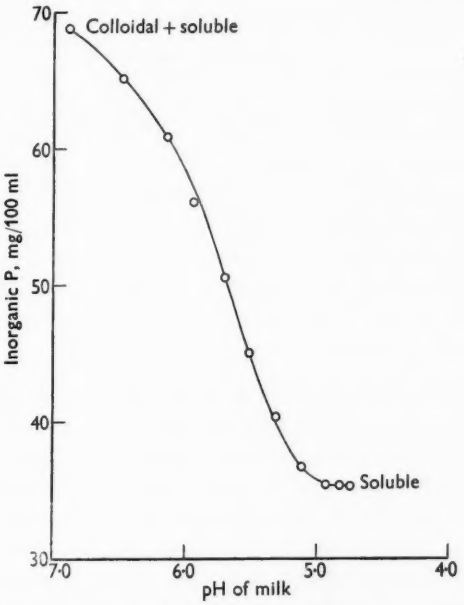

Fig. 1. Effect of acidification and subsequent dialysis (against unaltered milk) on the inorganic phosphate content of milk.

RESULTS

(1) *Composition of the colloidal phosphate as determined analytically and by oxalate titration*

Preliminary determinations of the calcium and inorganic phosphate contents of four milks and their corresponding colloidal phosphate-free milks revealed an average [Ca]/[P] ratio of 1·68 for the colloidal phosphate. This value agrees with that found by Evenhuis & de Vries (1956*b*) for the phosphate adsorbed from heated milk by finely divided solids, and considered by them to be hydroxyapatite. Five further determinations of the same kind, this time estimating [Ca + Mg] instead of [Ca], gave an almost identical average of 1·67 for the [Ca + Mg]/[P] ratio (Table 1). Clearly the magnesium content of the colloidal phosphate is negligible, as might

be expected, and accordingly in Tables 1–3 the ratio is referred to as [Ca]/[P], although it is determined as [Ca+Mg]/[P]. Determinations of the [Ca]/[P] ratios by the oxalate titration for three of these samples yielded, on the other hand, the much lower average value of 1·41 (Table 1) which is in good agreement with the results obtained previously for milk using this method (Pyne & Ryan, 1950). In marked contrast to the results given by direct analysis of the milk and corresponding colloidal phosphate-free milk (which, considered in isolation, could be interpreted as indicating the presence of hydroxyapatite), these oxalate titration results point once more to the presence in milk of a colloidal phosphate corresponding mainly to tricalcium phosphate but, as usual, with some admixture of the dicalcium salt.

Table 1. *The composition of the colloidal phosphate obtained from analysis and from oxalate titration of milk and of colloidal phosphate-free milk*

	Milk		Colloidal phosphate-free milk		[Ca]/[P] ratio in colloidal phosphate	
	Ca+Mg (*a*)	Inorganic P (*b*)	Ca+Mg (*c*)	Inorganic P (*d*)	By analysis $(a-c)/(b-d)$	By oxalate method
Expt.	m-mole/l.					
1	35·8	19·6	20·9	10·9	1·71	1·38
2	39·5	22·3	20·1	10·8	1·69	1·43
3	35·8	23·7	18·4	13·0	1·63	1·42
4	38·3	23·5	20·2	12·6	1·66	—
5	37·3	23·2	18·9	12·1	1·66	—
				Average	1·67	1·41

The question immediately arises—what is the origin of the marked divergence in phosphate composition furnished by the two methods and which method, if either, determines it correctly? Assuming for the moment that both methods are trustworthy, and there seems no reason to doubt this, the discrepancy observed could arise from the presence in milk of another acid-soluble calcium salt, perhaps associated with the phosphate. Passage into solution and loss of such a constituent during preparation of the colloidal phosphate-free milk might readily occur, and if so, could lead to overestimation of the calcium attributed to calcium phosphate proper, and thus to an unduly high [Ca]/[P] ratio for this phosphate determined analytically. On the other hand, the [Ca]/[P] ratio calculated from the results of oxalate titrations might in such circumstances remain practically unaffected by the presence of the additional calcium salt. In that event the oxalate titration might well throw more light on the nature of the colloidal phosphate actually present.

The acid-soluble colloidal calcium salt needed to account for those discrepancies is not, in fact, far to seek. In the form of colloidal calcium citrate it has long been recognized as a constituent of milk, if rather a minor one. Recent work of Verma & Sommer (1957) and of White & Davies (1958) suggests, however, that colloidal citrate may form a fairly significant fraction—some 10%—of the total milk citrate. In such amounts the presence of colloidal citrate could go far to account for the discrepancies between the results obtained by analysis and oxalate titration.

(2) *Influence of colloidal calcium citrate on the composition assigned to the colloidal phosphate*

Determinations of citrate in seven milks and corresponding colloidal phosphate-free milks showed that about 11 % (8–14 %) of the milk citrate is present in association with the colloidal phosphate, a proportion similar to that reported as colloidal citrate by Verma & Sommer (1957) and White & Davies (1958). During the preparation of colloidal phosphate-free milk this citrate naturally passes into solution. If the average colloidal citrate is taken as 1·2 m-mole/l., which is its mean value from the present experiments, the quantity of calcium combined therewith will approximate to 1·8 m-mole/l. if the citrate is represented as the tertiary salt. Corrected for this citrate calcium the [Ca]/[P] ratio of 1·67 calculated from the analytical data of Table 1 becomes 1·50, in definitely better agreement with the average ratio, 1·41, calculated from the results of oxalate titration.

The possibility remains that some of the difference between these ratios arises from neglect of a small proportion of magnesium phosphate as a component of the colloidal phosphate, and some perhaps from slight inherent inaccuracies in the oxalate technique. Neither contribution is thought to be very significant however. Thus solubility considerations make magnesium phosphate an unlikely component of the colloidal phosphate of milk, a conclusion confirmed, as already pointed out, by the virtual identity of the [Ca]/[P] and [Ca + Mg]/[P] ratios for the colloid, namely 1·68 and 1·67 respectively. (Colloidal magnesium is presumably mainly present in milk in combination with casein.) The oxalate titration would appear also to be a reasonably accurate procedure. Thus identical results were obtained with this method both as normally conducted and when the amount of oxalate used was doubled and the time of interaction greatly extended.

The [$Citr^{-}$]/[Ca] ratio of the colloidal phosphate has been found from the above analyses of milk and colloidal phosphate-free milk to average 1/15 (1/13–1/17). That this citrate is intimately combined with the colloidal phosphate in milk is shown by the impossibility of removing it by dialysis of milk against an excess of an approximately saturated calcium phosphate solution of the same pH. After dialysis of the milk for 96 h, with 24 h replacement of the phosphate by fresh solutions, no significant change in either the total or the oxalate determined [Ca]/[P] ratios of the colloidal phosphate could be observed. In two experiments these ratios, which before dialysis had been 1·70, 1·69, and 1·38, 1·43 respectively retained, after dialysis, the virtually unchanged values of 1·68, 1·69, and 1·39, 1·43 respectively; the persistence of citrate in the colloidal phosphate was moreover confirmed by analysis.

(3) *Composition of the precipitates formed on neutralizing natural and artificial acid milk sera*

Evenhuis & de Vries (1957) have recorded a [Ca + Mg]/[P] ratio of about 1·60 for the phosphate which is precipitated when acid whey is neutralized. This observation is one of the bases of their suggestion that a similar ratio might also characterize the colloidal phosphate of raw milk. In similar experiments with almost undiluted isoelectric milk serum neutralized with 2N-NaOH we have obtained both at 15 and 40 °C almost the same ratio as found for the colloidal phosphate of milk, i.e. 1·67.

If, however, as has been seen, the presence of colloidal citrate in milk can lead to the overestimation of the analytically determined [Ca]/[P] ratio of the colloidal phosphate of the milk, then probably the presence of citrate in whey will likewise lead to the overestimation of this ratio in the whey neutralization precipitate. In this connexion Kuyper (1938) has shown that marked co-precipitation of citrate with calcium phosphate can occur on neutralizing certain acid calcium phosphate—citrate mixtures. In view of these considerations the influence of citrate on the composition and behaviour of the precipitate formed on neutralizing typical, mainly artificially prepared, acid-milk sera, has been studied. Neutralization was effected by slow addition, with rapid mechanical stirring, of 2N-NaOH.

Typical results are shown in Tables 2 and 3. The composition of the precipitates in both series of experiments was arrived at from analysis of the original serum and of the corresponding filtrate after precipitation; oxalate titrations were made on the centrifugally separated well-drained precipitates.

Table 2. *The effect of citrate on the composition of the precipitate formed on neutralizing solutions containing calcium, magnesium and phosphate in concentrations similar to those in milk serum*

	Composition of mixtures before neutralization			Composition of filtrates after neutralization			[Ca]/[P] ratio in precipitates	
	Citr⁻	Ca+Mg* (*a*)	P (*b*)	Ca+Mg (*c*)	P (*d*)	pH	By analysis $(a-c)/(b-d)$	By oxalate method
Mixture	m-mole/l.							
1	11·0	28·0	20·0	14·6	11·5	6·74	1·58	1·38
2	0·0	28·0	20·0	8·0	3·2	6·98	1·19	1·16
3	0·0	24·5	20·0	6·6	2·9	7·32	1·05	—
4	0·0	21·0	20·0	4·7	4·7	6·83	1·07	1·03
5	0·0	17·5	20·0	4·0	7·8	7·07	1·11	1·04

* Proportions of calcium and magnesium: [Mg]/[Ca] = 1/7.

Table 2 shows that the precipitate formed in the absence of citrate was largely dicalcium phosphate, irrespective of moderate variations of calcium plus magnesium content, and that its [Ca]/[P] ratio (range 1·05–1·19) was approximately the same whether determined by analysis or by oxalate titration. The precipitate formed in the presence of citrate, on the other hand, had the high [Ca]/[P] ratio of 1·58 analytically determined and the comparatively much lower ratio of 1·38 by oxalate titration, results immediately reminiscent of those furnished by the colloidal phosphate of milk.

Table 3 shows the effect of varying the citrate concentration on the composition of the precipitate formed on subsequent neutralization. In this series of experiments the concentration of calcium plus magnesium was adjusted to maintain an approximately constant calcium plus magnesium ion concentration. Inspection of this table enables the following conclusions to be drawn:

(*a*) In the absence of citrate the precipitate formed was confirmed as consisting mainly of dicalcium phosphate. Agreement between the results of analysis and oxalate titration was reasonably good here.

(*b*) In the presence of citrate there was a sharp alteration in the nature of the precipitate formed—a concentration of citrate as low as 0·5 m-mole/l. sufficing to cause a change in the phosphatic component to the predominantly tricalcic salt. At the lower citrate levels agreement between the [Ca]/[P] ratio determined analytically and that derived from oxalate titration continued to be good. As the citrate content increased, however, the two ratios began to diverge, and finally attained a difference comparable to that shown by the colloidal phosphate of milk.

Table 3. *The effect of various citrate concentrations on the composition of the precipitate formed on neutralizing solutions containing calcium, magnesium and phosphate*

	Composition of mixtures before neutralization			Removed from solution on formation of precipitate			Citr⁻ in precipitate as % of total citr⁻	[Ca]/[P] ratio in precipitates	
	Citr⁻	Ca+Mg*	P	Citr⁻	Ca+Mg (*a*)	P (*b*)		By analysis (*a*/*b*)	By oxalate method
Mixture	m-mole/l.								
1	0·0	20·0	20·0	0·0	16·4	14·5	—	1·13	1·08
2	0·47	20·5	20·0	0·39	17·1	12·3	83	1·39	1·41
3	0·93	21·0	20·0	0·44	17·7	12·4	47	1·43	1·41
4	2·80	23·0	20·0	0·76	18·5	12·6	27	1·47	1·40
5	4·67	25·0	20·0	1·36	18·0	12·0	29	1·50	1·40
6	9·34	30·0	20·0	1·74	17·6	11·2	19	1·57	1·36
7	14·01	35·0	20·0	1·96	17·4	10·6	14	1·64	1·35

* Proportions of calcium and magnesium: [Mg]/[Ca] = 1/7.

(*c*) The proportion of citrate present which was co-precipitated with phosphate was relatively great at low concentrations of citrate but declined as the concentration rose. At citrate and other ion concentrations comparable to those of isoelectric milk serum, some 16·5 % (14–19 %) of the citrate present was co-precipitated, giving to the precipitate in turn a [Citr⁻]/[Ca] ratio of about 1/10, that is, a value definitely higher than the 1/15 ratio found in the colloidal phosphate of milk (see (2) above).

(*d*) The changes brought about by entry of increasing amounts of citrate into the precipitate, namely, a steady rise in the [Ca]/[P] ratio determined analytically, and a slight fall in the ratio as determined from oxalate titration, followed a pattern which though immediately recognizable is not readily expressible by any single simple formula. These changes can be described in effect as approximately equivalent to a progressive replacement by $CaHCitr^-$ of the $CaHPO_4$ of a mainly tricalcium phosphate which itself is simultaneously tending to become more exclusively tricalcic.

The joint result of these various tendencies is the formation, at constituent concentrations comparable to those of isoelectric milk serum, of a precipitate whose analytical composition approaches that of a hypothetical citrate apatite of the type $3Ca_3(PO_4)_2$, $CaHCitr^-$; this may be alternatively, if more cumbrously, written as $2{\cdot}5Ca_3(PO_4)_2$, $CaHPO_4$, $0{\cdot}5Ca_3Citr_2^-$.

DISCUSSION

The results furnished by experiments on colloidal phosphate-free milk suggest that the so-called colloidal phosphate of milk should more properly be termed a colloidal phosphate—citrate. The intimacy of union of phosphate and citrate is demonstrated

by the impossibility of removing the citrate component from the whole, even on prolonged dialysis of milk against calcium phosphate solutions, as well as by the work of Kuyper (1938), in regard to calcium phosphate—citrate precipitates in general.

With a [Ca]/[P] ratio of about 1·67 determined analytically, and of about 1·41 determined by oxalate titration, the colloidal calcium phosphate—citrate behaves as if it possessed the approximate empirical formula of the hypothetical citrate apatite $3Ca_3(PO_4)_2$, $CaHCitr^-$. Its citrate content, however, is somewhat lower than this formula would indicate. The phosphate—citrate precipitates formed on neutralization of acid milk sera, natural or artificial, are essentially similar in composition to the colloidal phosphate—citrate, if perhaps somewhat less basic. They possess, however, a definitely higher citrate content, so that in this respect they conform more closely to the empirical formula set out above.

Citrate is known to occur in other biological phosphatic materials and consideration of one of these can perhaps throw light on the association of citrate with phosphate in milk. Thus fairly high contents of citrate have been observed to occur in bone (aconitic, malic, and succinic acids are also found but to a much smaller degree) and to co-precipitate with bone salts in experiments *in vitro* (Lees & Kuyper, 1957). Of certain pairs of acids studied in that work it can be seen that the hydroxy acid always co-precipitated more fully than did the corresponding or nearly corresponding non-hydroxy acid. This difference may perhaps be related to the relatively greater calcium binding powers of the former group.

The relatively lower citrate content of the colloidal phosphate of milk compared with phosphate—citrate complexes precipitated from milk sera has its origin no doubt in the fundamentally different conditions in which these materials are laid down. Obvious chemical differentiating factors which perhaps contribute to this result are the presence of calcium caseinate and perhaps also of appreciable amounts of calcium bicarbonate in the milk. Both these substances may in a sense compete with calcium citrate for sites in the phosphate—citrate complex of milk and may in fact provide minor constituents of this milk colloid. This point has not been examined.

Citrate, as noted in the present paper, and caseinate as shown by Eilers (1947), both incidentally substances of relatively high calcium binding power, possess the property of inducing formation of tricalcium phosphate in conditions where formation of the dicalcium salt might have been expected.

There remains the question of variability of composition of colloidal phosphate from one milk to another as demonstrated, for example, by the results of oxalate titration (Pyne & Ryan, 1950; White & Davies, 1958). Such variability seems to run counter to the relative fixity of the non-citrate or residual phosphate composition observed in the present work, and to which presence of citrate and caseinate appear to contribute. The methods of investigation hitherto employed may be at fault here. Oxalate titration, though useful, is perhaps not a sufficiently sensitive method for the examination of variability of composition; the ideal milk serum for the determination of soluble phosphate is not yet fixed. Thus milk dialysate and ultrafiltrate, hitherto mainly used, differ markedly, for instance, from rennet whey in this respect, as has frequently been pointed out (Pyne, 1940). The similarity in the phosphate content of rennet whey and of colloidal phosphate-free milk, the latter being obtained

under conditions such that the influence of the Donnan effect on its composition will be negligible, suggests that either preparation can be used to obtain an accurate value for the soluble phosphate content of milk. Nevertheless, these matters appear to need re-examination in the light of the present technique and results.

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A quantitative evaluation of some factors affecting the non-fatty solids of cow's milk

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Summary. Ten farms in the county of Durham took part in a field study of the effects of feeding and of udder disease on the level of non-fatty solids (S.N.F.) in milk. Statistical analysis of the resulting data showed that age, pregnancy, season of the year, and total cell count affected the percentage of S.N.F. and that these effects were additive and independent of each other. No effect associated with nutritional changes could be demonstrated.

The principal effects of the factors, each one freed from effects of other factors, were as follows:

	S.N.F. %
(1) Decline with age from 1st and 2nd lactations to 5+ lactations	0·47
(2) Mid-lactation fall	0·25
(3) Reduction associated with high cell count	0·15
(4) Rise from winter to spring	0·13

Herds in which S.N.F. had been consistently low over a period of years were compared with herds in which S.N.F. had been satisfactory. Analysis of the data showed that about 70 % of the difference in S.N.F. between these groups could be accounted for by differences in age of cow, stage of lactation, cell count and breed.

There was some evidence of a residual effect following clinical mastitis that could not be accounted for by residual high cell counts.

The within-cow regression of S.N.F. on log cell count calculated from the Durham data and from van Rensburg's data was on both occasions negative.

The implications of these findings are discussed, particularly in relation to advisory work.

The data used in this study resulted from an investigation on ten farms in County Durham, in which a number of specialist advisory officers co-operated. The main object of the investigation was to study the effects of alterations in feeding, and of udder disease on the non-fatty solids (S.N.F.), but the analysis of the results necessarily required a statistical study of inter-relationships with other factors known to affect S.N.F.—age, stage of lactation, season of the year and pregnancy (see review

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by Bailey, 1952*a*). The separate and combined effects of these various factors on the S.N.F. of the bulk milk of a herd, and their relative importance, have therefore been evaluated. Published information of this type has been lacking.

FIELD INVESTIGATIONS

Seven of the ten herds had a history of low S.N.F. (problem farms) and three were known to have no low S.N.F. history and to have a satisfactory standard of management (control farms). The only other criterion for selection of farms was the willingness of the farmers to co-operate. On all the farms there was a total of approximately 187 cows.

The first 9 months of the investigation (August 1956–April 1957) consisted of a control period, when each herd continued under its normal management. For the remainder of the investigation (April 1957–July 1958) advice was given on various aspects of management. Each farm was visited at approximately fortnightly intervals, when information was obtained on details of feeding, cropping, breeding, herd numbers and changes, age of cows, stage of lactation, milk yield, calvings, drying off, service and disease.

Purchased concentrates and all feedingstuffs, except those with standard values, were sampled and examined. Grazing was periodically examined and its feeding value assessed.

During the advisory period, rations were prescribed for each cow individually on all the seven problem farms, on the basis of the quantities and qualities of the available food. These rations were based on Woodman's standards (Woodman, 1952). A detailed examination of the starch equivalent and digestible protein levels actually fed revealed some deficiencies and surpluses in actual practice. Deficiencies were mainly of starch equivalent, but occasionally of digestible protein. Most of the instances of deficiency in starch equivalent occurred in the winter feeding period before Christmas. The position during the summer was difficult to establish although efforts were made, both by analysis and observation, to assess the productivity of the grazing available. In general the summer feeding, except of high-yielding cows, appeared to be adequate.

The milk from each cow was sampled once each month, morning and evening samples being mixed in proportion to respective yields (udder samples). During the investigation milk samples were drawn from individual quarters in three herds (quarter samples). All milk samples were examined for butterfat, S.N.F., total cell count and California mastitis test reaction. All the S.N.F. results referred to in this report were obtained by subtracting the Gerber butterfat percentage from the total solids percentage determined gravimetrically.

The term 'clinical mastitis' is used to denote instances reported by the co-operating farmers of cows having developed clinical symptoms. It was not practicable to have the farmers' diagnoses confirmed. Samples taken during the remainder of the lactation from these cows are discussed under a separate heading in this report. Some cows probably developed slight symptoms which either passed unnoticed or were not reported.

Since total cell counts in the milk are believed to be an index of a degree of udder

inflammation even in the absence of clinical symptoms, these counts were included in the investigation to indicate whether the subclinical condition affected the S.N.F. At the start of the investigation total cells in samples were counted by examining fifty microscopic fields. This was continued throughout with quarter samples, but to reduce the volume of laboratory work, a modified test in which fifteen fields were examined was subsequently adopted for udder samples.

Results were classified as follows:

	Udder samples (cells/ml) $\times 10^{-3}$		Quarter samples (cells/ml) $\times 10^{-3}$
Low	100	Low	250
Medium	101–500	Low—medium	251–600
High	501+	High—medium	601–1000
		High	1000+

The definitions of the three categories for udder samples were based on the work of Waite & Blackburn (1957). Laing & Malcolm (1956) suggested that a total cell count of 250000/ml in a quarter sample of foremilk should be regarded as suspect. The categories above this basic low standard were adopted for the purpose of this investigation to simplify the study of the relationship between cell count and S.N.F. In view of the association shown by Waite & Blackburn (1957) between total cell count and polymorph cells, differential tests were not carried out.

During the course of the investigation the total cell counts obtained from three herds indicated that there was probably a high incidence of udder disease in these herds. The Veterinary Investigation Officer examined quarter samples of milk from each of these three herds bacteriologically.

Samples were taken at all seasons of the year. Those taken during the 2 months commencing 2 weeks after spring grazing started were defined as spring samples, and those taken during the 3 months 15 December–15 March as winter samples.

STATISTICAL ANALYSES

The quantities of data available from udder samples are summarized in Table 1. Farms 8–10 provided the 'control' herds. Since there were four factors to be studied —age of cow, season, cell count and stage of lactation—the first step was to summarize

Table 1. *Numbers of samples analysed and herd sizes*

Herd no.	From cows without mastitis	From cows with clinical mastitis	Total samples	No. of cows in herd
1	378	66	444	20
2	52	13	65	8
3	485	0	485	26
4	121	41	162	10
5	312	0	312	15
6	423	21	444	22
7	250	77	327	16
8	203	7	210	11
9	559	25	584	30
10	393	34	427	29
Total	3176	284	3460	—

the data and obtain the means for each factor and for the 2-factor tables. The set of 3176 observations was punched on paper tape and summarized on an electronic computer. The other 284 were punched on cards and summarized on a Hollerith tabulator.

A. *Samples from cows without clinical mastitis*

The sample means and the numbers on which these are based are given in Table 2.

All four factors appeared to influence the S.N.F., the most potent being age. Winter results were lower than spring results in both years, and the results in the first year were lower than those in the second year. The two-way tables suggested that each factor was acting independently of the others; the decline with advancing age, for example, could be seen in samples from all stages of lactation and the drop in S.N.F. in mid-lactation occurred at all ages.

Table 2. *Means of the data* (3176 *samples*) *from cows free from clinical mastitis at each level of each factor*

Factor	Mean S.N.F., %	No. of samples
Age, lactation		
no. 1	8·69	1173
2	8·63	747
3	8·41	507
4	8·29	342
5	8·28	236
6+	8·06	171
Season		
1956–57: Winter	8·40	389
Spring	8·52	480
1957–58: Winter	8·46	497
Spring	8·56	527
All other samples	8·56	1283
Cell count		
Low	8·59	1313
Medium	8·54	1339
High	8·31	524
Stage of lactation		
Months 1	8·66	458
2	8·39	406
3	8·42	408
4	8·44	383
5	8·43	350
6	8·49	330
7	8·50	283
8	8·67	222
9	8·71	149
10+	8·78	179

The numbers in the subclasses were very different; the means for each factor in Table 2 are therefore biased to some extent, but unbiased estimates were obtained by the method of fitting constants, a programme for the electronic computer being available for this purpose. An example of the unequal distribution of sample numbers is given in Table 3, which shows that the old cows tended to have high cell counts and therefore that the mean of 8·31 % S.N.F. for samples with high cell counts in Table 2 is probably too low and is partly reflecting an age effect. Conversely,

the mean of 8·06 % for the old cows is too low because 45 % of the samples had a high cell count.

The data were grouped before analysis into three levels of each factor, giving the results shown in Table 4. Each effect shown in this table is freed from the effects of all other factors. Age is still the most potent factor. The association of high cell count with a reduction in S.N.F. (0·15 %) remains when age and other effects are eliminated.

Table 3. *Part of a 2-factor table of sample numbers*

Lactation no.	Cell count group			Total	% high cell counts
	Low	Medium	High		
1	659	421	93	1173	7·9
6+	27	67	77	171	45·0

Table 4. *Effects of the three levels of each factor, shown as deviations from the mean* (8·52) *of all samples*

Lactation no.	Deviation	Season	Deviation	Cell count	Deviation	Month of lactation	Deviation
1, 2	+0·12	Winter	−0·09	Low	+0·03	1, 8+	+0·16
3, 4	−0·14	Spring	+0·04	Medium	+0·02	6, 7	−0·03
5+	−0·30	Others	+0·02	High	−0·12	2–5	−0·09

Exact tests of significance of the effects of the four factors would be impossibly complicated because there were varying numbers of successive observations which could not be assumed to be independent. However, testing the main effects against the interactions should eliminate most of the effect of the correlations between successive observations. The analysis of variance on this basis showed, first, that the 2-factor interaction mean square was about equal to the mean square for the remaining interactions (3- and 4-factor) and secondly that all four mean squares for main effects were highly significant when tested against the interaction mean square.

It can therefore be stated that high cell count was associated with a significant reduction in S.N.F., whatever the age or stage of lactation or season of the year; this does not constitute a proof that reduction of the cell count would result in an increase in S.N.F., though such a result seems probable.

After adjustment for the effects of the other three factors, the changes associated with advancing lactation remained substantially those shown by the unadjusted means of Table 2. The rise late in lactation was suggestive of an effect of pregnancy, and a section of the data was re-examined from this point of view. The data available were monthly samples from cows conceiving from the fifth month of lactation onwards. Only sets that were complete for the first 9 months of lactation were used, with the result that rather small numbers of cows were represented. The means at each month of lactation are given in Table 5. There is considerable fluctuation from month to month in data based on such small numbers; however, regression lines fitted to each set of nine means have slopes that decrease steadily as the date of conception becomes further removed from the previous calving date (Table 6). This is consistent with the results of Bartlett (1934) and Bailey (1952*b*), who showed that after the sharp fall in early lactation there was a rise later in lactation starting after

conception. This rise did not occur in the absence of conception. This table also shows the drop from the initial value that is likely to occur by the ninth month of lactation. It seems that the S.N.F. of cows conceiving after the sixth month will be very likely to fall below the legal minimum (8·50 %) in the ninth month of lactation.

Table 5. *Effect of calving interval on non-fatty solids; the mean non-fatty solids for cows conceiving in the 5th, 6th, ..., 12th months of lactation*

Month of lactation in which conception occurred	No. of cows	Month of lactation								
		1	2	3	4	5	6	7	8	9
5	26	8·66	8·44	8·44	8·52	8·36	8·41	8·62	8·48	8·51
6	8	8·53	8·36	8·32	8·34	7·96	8·38	8·08	8·44	8·35
7	4	8·36	7·88	8·08	8·46	8·34	8·06	7·82	7·61	7·89
8	8	8·71	8·46	8·46	8·35	8·40	8·25	8·16	8·26	8·06
9–12	7	8·53	8·75	8·45	7·82	8·31	8·08	8·00	7·64	7·76

Table 6. *Slopes of regressions of percentage non-fatty solids on month of lactation fitted to the data of Table 5*

Conception month	Slope, % S.N.F. per month	Expected drop in 9 months
5	−0·004	−0·04
6	−0·015	−0·14
7	−0·060	−0·54
8	−0·065	−0·58
9–12	−0·118	−1·06

B. *Comparison of problem and control farms*

A similar analysis of the data from the problem and control farms showed that the effects of the four factors were closely similar in both groups, and this analysis provided a means of estimating the extent to which the differences between the two groups could be accounted for by differences in the distribution of age, cell count, etc., in the two groups.

For this analysis, low and medium cell-count data were combined because the estimates of their effects did not differ appreciably. For the same reason the 'spring' and 'others' classes were combined, reducing the seasonal comparison to winter versus remainder. The data in Table 7 are the constants, which are deviations from the general mean, and the differences between these constants are directly comparable from one group of farms to the other.

The decline in S.N.F. with advancing age was essentially the same in both groups of herds. Differences in the absolute values of the two sets of constants are caused largely by differences in the age distributions of cows in the two groups, there being many more samples from old cows in the group of problem herds.

The winter effect was evident only in the problem herds which suggests that winter feeding in these herds was defective in some way. The cell count effect was slightly greater in problem than in control herds. This difference may perhaps be an artifact caused by the crude grouping of cell-count data. A high cell count was defined as 'over 500 000 cells per ml'. The mean cell counts in this category might well have

been much higher for problem than for control herds, and would therefore be expected to be associated with a greater reduction in S.N.F. in the problem herds.

As the effects of the four factors are additive, the general mean plus sums of the relevant constants provides values to be expected for a cow of a given age at a given stage of lactation, when cell count is low and the sample is taken outside the winter months. Thus, for the problem herds, the expected value for a cow in lactations 1 and 2 and in the 6th or 7th month of lactation is $8{\cdot}40 + 0{\cdot}14 + 0{\cdot}05 + 0{\cdot}03 - 0{\cdot}04 = 8{\cdot}58$ and for a cow from the control herds, $8{\cdot}73 + 0{\cdot}06 + 0 + 0{\cdot}01 - 0{\cdot}03 = 8{\cdot}77$. Expected values for the nine age × stage of lactation groups are shown in Table 8 and the numbers of samples in Table 9. The latter table shows that proportionately many more samples came from young cows in control than in problem herds. If the expected

Table 7. *Constants fitted for the four factors to data from problem and control herds*

(General means: problem herds 8·40, control herds 8·73)

	Problem	Control
Lactation nos. 1, 2	+0·14	+0·06
3, 4	−0·11	−0·13
5+	−0·22	−0·30
Diff. (first minus last)	+0·36	+0·36
Season: Winter	−0·13	−0·01
Others	+0·05	0·0
Diff. (first minus last)	−0·18	−0·01
Cell count: Low and medium	+0·03	+0·01
High	−0·12	−0·09
Diff. (first minus last)	+0·15	+0·10
Month of lactation: 1, 8+	+0·18	+0·13
6, 7	−0·04	−0·03
2–5	−0·09	−0·08

Table 8. *Expected percentage non-fatty solids for samples with low or medium cell count taken outside the winter months*

Lactation no.	Problem herds. Stage of lactation			Control herds. Stage of lactation		
	1, 8+	2–5	6, 7	1, 8+	2–5	6, 7
1, 2	8·80	8·53	8·58	8·93	8·72	8·77
3, 4	8·55	8·28	8·33	8·74	8·53	8·58
5+	8·44	8·17	8·22	8·57	8·36	8·41

Table 9. *Numbers of samples taken from problem and control herds, showing age by stage of lactation distribution*

Lactation no.	Problem herds. Stage of lactation					Control herds. Stage of lactation				
	1, 8+	2–5	6, 7	Total	% samples	1, 8+	2–5	6, 7	Total	% samples
1, 2	368	521	181	1070	53	291	376	183	850	74
3, 4	156	322	128	606	30	70	123	50	243	21
5	103	186	56	345	17	20	27	15	62	5
	627	1029	365	2021		381	526	248	1155	
% samples	31	51	18			33	46	21		

values for the two groups be weighted by the numbers of samples from control herds, the expected means based on the same population of samples are obtained.

These weighted means are 8·56 for problem herds and 8·74 for control herds. When the two groups of herds are compared on the same basis, the original difference between the means (0·33) is thus reduced to 0·18. The comparison is complicated by breed differences, for the problem herds contained 10 Shorthorns, 99 Friesians and 8 mixed, whereas the controls were 11 Shorthorns, 30 Ayrshires and 29 Friesians. Weighting the breed averages for S.N.F. percentage (Ministry of Agriculture, Fisheries and Food, 1957) by numbers of cows, giving the mixed herds the same percentage of S.N.F. as Shorthorns, the weighted means are 8·60 for problem herds and 8·68 for control herds. Thus out of the remaining difference between problem and control herds (0·18), 0·08 can be accounted for by breed differences, leaving 0·10 unaccounted for.

Table 10. *Effects of the four factors in problem and control herds in two successive years*

Effects of season

				No. of samples		
	Winter	Spring	Mean	Winter	Spring	*P*
Problem, 1956–57	−0·10	+0·09	8·33	242	283	<0·01
1957–58	−0·11	+0·10	8·40	320	354	<0·001
Control, 1956–57	+0·02	−0·01	8·68	147	197	—
1957–58	+0·02	−0·01	8·72	177	173	—

Effects of age

	Lactation no.			No. of samples			
	1, 2	3, 4	5+	1, 2	3, 4	5+	*P*
Problem, 1956–57	+0·13	−0·15	−0·11	275	143	107	<0·01
1957–58	+0·14	−0·08	−0·26	334	233	107	<0·001
Control, 1956–57	+0·06	−0·16	−0·34	262	67	15	<0·01
1957–58	+0·04	−0·04	−0·29	247	86	17	<0·01

Effects of cell count

			No. of samples		
	Low and medium	High	Low and medium	High	*P*
Problem, 1956–57	+0·03	−0·08	403	122	—
1957–58	+0·03	−0·15	554	120	<0·01
Control, 1956–57	+0·02	−0·09	288	56	—
1957–58	+0·00	−0·06	325	24	—

Effects of stage of lactation

	Month of lactation			No. of samples			
	1, 8+	6, 7	2–5	1, 8+	6, 7	2–5	*P*
Problem, 1956–57	+0·14	+0·02	−0·07	302	138	85	<0·05
1957–58	+0·17	−0·10	−0·08	328	226	120	<0·001
Control, 1956–57	+0·18	−0·05	−0·09	171	100	73	<0·001
1957–58	+0·14	+0·06	−0·10	178	107	65	<0·001

P = probability that observed effect would occur in random sampling.

The important feature of this analysis is that 70 % of the difference between the mean % S.N.F. for these two groups of herds could be accounted for by observed differences in age and breed of cow and in the cell count. The method used for correcting for breed differences assumes that the cows in these herds were genetically representative of their own breeds and that the published data are also representative. Since these assumptions cannot be checked, the correction for breed differences is arbitrary and merely an indication that breed could account for an appreciable part of the difference between the two groups.

The unadjusted means of Table 2 have higher values for both winter and spring samples in 1957–58 than in 1956–57. It was necessary therefore to discover whether these higher values reflected differences in the effects of one or more of the four factors, or were produced by differences in the numbers of samples in the various subgroups. Results of the analysis (Table 10) showed that the effects of the four factors were of the same order in both years. The effects of season were almost identical, confirming that there was a real effect only in the problem herds. In the problem herds, the 5+ age group was associated with a greater depression of S.N.F. in 1957–58 than in 1956–57. It may be that this group had a higher average age in the second year than in the first. There were no consistent differences between the years in the effect of cell count and stage of lactation. The main reason for the increase from 8·33 to 8·40 % S.N.F. in the problem herds appears to be the increase in numbers of samples from cows in the younger age groups, with a consequent increase in numbers of samples with low and medium cell counts. The slight increase from 8·68 to 8·72 % S.N.F. in control herds was associated with an increase in number of samples with low and medium cell counts and a decrease in numbers with high cell counts.

C. *Samples from cows with clinical mastitis*

Constants for age, season, cell count and stage of lactation were fitted to the 284 observations relating to cows for the remainder of their lactations after they had been affected by clinical mastitis. These constants were very similar to the constants for unaffected cows and can be compared in Table 11. The mean for the 284 samples was 8·19 % and that for the unaffected cows 8·52 % S.N.F., but 37 % of the samples from affected cows were in the highest age group, compared with 13 % of the samples from unaffected cows. Comparing the two groups by the method used for comparing problem and control herds, the adjusted means were 8·39 % S.N.F. for affected cows and 8·58 % for unaffected cows. The difference (0·19 %) may be a residual effect of clinical mastitis that is additional to the effect associated with increase in cell count. There were, however, differences between the two groups such as differences in breed which have not been taken into account in this analysis. Nearly half the original difference (0·33 %) between the means of the two groups has been accounted for by the four factors investigated. In Table 12 it can be seen that samples from cows that had been affected by clinical mastitis in the first and second lactations had closely similar proportions of low, medium and high cell counts to the proportions in samples from the oldest group of unaffected cows. These proportions did not differ in samples from the group of oldest cows. This might imply either that young animals in which the cell count is high tend to contract mastitis or that clinical mastitis tends to induce senile changes in the young udder.

Table 11. *Constants for each level of the four factors, comparing samples from cows affected with clinical mastitis with samples from the unaffected cows*

	Month of lactation			
	1, 8+	6, 7	2–5	Mean
Non-mastitis	+0·16	−0·03	−0·09	8·52
Mastitis	+0·12	+0·09	−0·10	8·19
Diff. between means	0·37	0·22	0·34	0·33

	Cell count			
	Low	Medium	High	Mean
Non-mastitis	+0·03	+0·02	−0·12	8·52
Mastitis	+0·11	+0·06	−0·13	8·19
Diff. between means	0·25	0·29	0·34	0·33

	Age (lactation no.)			
	1, 2	3, 4	5+	Mean
Non-mastitis	+0·12	+0·14	−0·30	8·52
Mastitis	+0·15	−0·04	−0·11	8·19
Diff. between means	0·30	0·23	0·15	0·33

	Season		
	Winter	Others	Mean
Non-mastitis	−0·09	+0·03	8·52
Mastitis	−0·06	+0·02	8·19
Diff. between means	0·31	0·34	0·33

Table 12. *Percentage of samples in each age group with low, medium and high cell counts*

Lactation no.	Samples from unaffected cows				Samples from affected cows			
	Low	Medium	High	Total	Low	Medium	High	Total
1, 2	51	40	9	100	24	40	36	100
3, 4	29	47	24	100	21	41	38	100
5+	20	41	39	100	20	38	42	100

D. *Quarter samples*

Age, season and stage of lactation effects are eliminated when the cell count—S.N.F. relationship is calculated from differences between the quarters of the same cow. Quarter samples had been taken on three farms, two of which were severely affected with clinical mastitis, but only those sets could be used where the cell counts from different quarters fell into different groups. With the grouping of cell counts used, four quarter samples from the same cow all having low cell counts contribute no information about the cell count—S.N.F. relationship.

The problem of the correlation between successive observations on the same cow was avoided by using only one set of results from each cow. Altogether forty-two sets of quarter samples were suitable for analysis. All counts had been grouped into four

classes, namely: low, low medium, medium high and high. Half the samples fell into the low group and only one-eighth into the high group.

Taking as the mathematical model

$$y_{ij} = m + c_i + d_j + e_{ij},$$
$$i = 1, 2, \ldots, 42, \quad j = 1, 2, 3, 4,$$

where m represents a mean, c_i an effect associated with cow i, d_j an effect associated with cell count group j, and e_{ij} representing residual error, 47 equations result, but these can easily be reduced by substitution to a set containing only the d's, which were solved by making $d_1 = 0$. The values obtained were:

Cell count	Constant (% S.N.F.) with standard error
Low (d_1)	0
Low medium (d_2)	$-0{\cdot}030 \pm 0{\cdot}101$
Medium high (d_3)	$-0{\cdot}162 \pm 0{\cdot}240$
High (d_4)	$-0{\cdot}322 \pm 0{\cdot}177$

The within-cow estimation gives a relationship between cell count and S.N.F. essentially similar to that obtained from udder samples. None of the constants, however, reaches the standard level of significance ($P = 0{\cdot}05$), because of the small numbers, particularly in the group with high cell counts, nor does the regression of the d's on increasing cell count attain significance.

Table 13. *Regressions of percentage non-fatty solids on log cell count calculated from van Rensburg's data*

Lactation no.	Days after calving	Regression coefficient ± S.E. (% S.N.F.)	Mean log cell count	Mean % S.N.F.
2	116	$-0{\cdot}143 \pm 0{\cdot}048$	5·43	8·47
	144	$-0{\cdot}191 \pm 0{\cdot}037$	5·41	8·43
3	88	$-0{\cdot}158 \pm 0{\cdot}050$	5·73	8·38
	144	$-0{\cdot}055 \pm 0{\cdot}067$	5·48	8·39
	200	$-0{\cdot}122 \pm 0{\cdot}055$	5·76	8·51
	Mean	$-0{\cdot}134$	5·56	8·44

The detailed results published by van Rensburg (1947) provide a means of supplementing this analysis by the calculation of regression coefficients which express the within-cow relationship between cell counts and % S.N.F. for cows free from clinical mastitis. The data give details for ten cows at ten stages of lactation in the first three or four lactations. Cell counts are not given for all samples, but five complete sets were analysed to obtain the regression of % S.N.F. on log cell count. For the analysis, a cow was regarded as a single replicate of a 2×2 factorial experiment, the factors being left versus right side of the udder and front quarters versus hind quarters. The regression coefficients were calculated from residual mean squares. The five regression coefficients with their standard errors (Table 13) are consistently negative and closely similar, and the mean value ($-0{\cdot}13$) implies that in these cows a tenfold increase in cell count was on the average associated with a drop of 0·13 % S.N.F. This is somewhat less than the difference between 'low' and 'medium high' given above, where approximately a fourfold increase in cell count was associated

with a drop of 0·16 % S.N.F. The expected drop associated with a fourfold increase in van Rensburg's cows was 0·08 % S.N.F. and this is certainly consistent with our data, bearing in mind the standard errors of the constants.

DISCUSSION

The most important factors affecting the level of S.N.F. in this investigation were the age of cow and the effect of pregnancy in reversing the lactational decline in S.N.F. The statistical investigation of the effect of pregnancy was undertaken at a later stage of the analysis of the data and is based on relatively small numbers of samples. A thorough study of this point would require a complete re-analysis of the data, but the effect is so marked that further investigation could scarcely cause a substantial change. It seems likely that a detailed investigation on the effect of the intervals between calvings might throw further light on, and perhaps account for, an even greater proportion of the observed differences between the mean S.N.F. of groups of farms. That the normal mid-lactation rise depends on conception is a fact that could be important in many herds.

Age of cow was also important, partly because there appeared to be a natural decline in S.N.F. with increasing age, and partly because in older animals there is a higher incidence of udder disease. Thus the young fertile herd may generally be expected to have little trouble with S.N.F., whereas a herd of old cows conceiving late in lactation is much more likely to produce milk below the legal standard.

It is generally accepted that high cell counts indicate some degree of udder inflammation. This need not be associated with infection, although it may be the product of a condition of susceptibility to infection. The statistical analysis shows that high cell counts were associated with a decrease of 0·15 % S.N.F. in cows not affected by clinical mastitis during the observed lactation. It is shown that there appeared to be an effect of mastitis additional to the effect that could be attributed to high cell counts amounting to a further reduction of 0·19 %.

High cell counts were, in all sets of samples investigated, associated with reduced S.N.F., although some individual samples did not show this association. It is clear that the reasons for such exceptions require investigation.

The three farms with the greatest proportion of high cell counts had the greatest degree of infection and the lowest S.N.F., although the level of S.N.F. is complicated by the effects of the other factors. On two of these farms high cell counts persisted after infection was removed by antibiotic therapy. High cell counts occurred both in milk produced by cows that had been cured of mastitis and in milk from cows that had not been affected. Although this shows that high cell count (in the absence of mastitis) is associated with low S.N.F., and that mastitis is associated with high cell counts and even lower S.N.F., it appears that mastitogenic organisms do not always cause the high cell counts. The reason for the persistence of high cell counts after the removal of these organisms seems to be a matter for further investigation, as also is a possible relationship between milking technique and total cell count.

The difference between winter and spring samples occurred only in the problem herds and was quantitatively similar in both years, in spite of all the advice on feeding standards given during the second year. It could be argued therefore that the low

winter S.N.F. was not a nutritional effect, or that it was, but was not altered by increasing the levels of dietary energy and protein. However, there was no significant difference between spring and winter S.N.F. in the control farms where the levels of feeding were known at all times, and had for some years been of a high standard. Feeding on most of the problem farms was known to have been poor for some years previously; even during the advisory period grazing was sometimes deficient in quantity and/or quality, and winter feeding, especially for some of the high yielding cows, did not always reach the level advised.

However, the advice given resulted for the most part in substantial increases in the levels of protein and starch equivalent fed on these farms which, if these were the nutritional factors related to the low winter S.N.F., should have shown an appreciable effect. The fact that there was no suggestion of a response to increased dietary protein and starch equivalent suggests that some other factor, possibly also nutritional, was responsible for the winter decline. It should be noted that this seasonal effect was numerically the smallest of all the factors investigated.

In a herd where there is a persistent problem of low S.N.F. we would suggest that an advisory officer should determine the ages and calving intervals of the cows. Either of these might provide an immediate point of attack. In a young herd with a normal average calving interval a high incidence of mastitis might be sufficient to account for an S.N.F. problem. We cannot assert that removal of the mastitis infection would improve the S.N.F. in the same lactation. The California mastitis test or the Whiteside test might well be substituted for the more laborious total cell count. Although we have no data on the effect of milking technique, we think that major faults should be looked for and corrected, since these may be contributing to high cell counts and creating susceptibility to mastitis. Since Robertson, Waite & White (1956) show that there is a strong genetic component affecting S.N.F. we would suggest that where age, fertility, mastitis and milking technique do not appear to be responsible for an S.N.F. problem, the advisory officer should assume that there may be a breeding effect and advise accordingly. Also, although the advice on feeding had no effect on the farms in this investigation, there may well be herds in which the correction of a gross deficiency would produce an increase in S.N.F. All these considerations will of course have to be weighed in the economic balance before the action appropriate to any particular farm is decided. A set of standards by which to assess the relative contribution of each factor would be a useful asset in advisory work. To be generally applicable, such a set would have to be derived from a larger number of farms situated in different parts of the country. The quantitative results of this study put the relative contribution of each factor in perspective; it would be unwise to regard them as standards, in view of the restricted source of the data.

This work was carried out under the general direction and with the encouragement of Mr R. Bruce, Regional Director, N.A.A.S. The chemical analyses and general supervision of advice on feeding were carried out by Mr N. Trinder, Nutrition Chemist. Mr C. H. Westwater, Regional Advisory Bacteriologist, undertook the cell count estimations, and Mr D. Buntain, Veterinary Investigation Officer, was responsible for the bacteriological examinations and veterinary work. The general

work of the investigation was carried out by the staff of the Milk Department. Miss Muriel E. Davis assisted with the statistical analysis.

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Identification of lactobacilli from Cheddar cheese by chromatography

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Summary. The technique of paper chromatography of bacterial extracts has been successfully applied to the routine examination of lactobacilli from cheese. One hundred and nine strains isolated on selective media have been grouped by their chromatogram patterns, and selected strains from these groups have been shown to have biochemical characteristics giving relationships which are in agreement with the chromatogram groupings. The results indicate that the technique can be used routinely for the rapid identification of strains of lactobacilli.

In problems involving the effects of bacterial activity, as for example the precise effects of bacterial enzymes in cheese ripening, it has been necessary to use biochemical tests or serological methods to identify individual strains. Naylor & Sharpe (1958*a*, *b*) have pointed out that it is desirable to identify lactobacilli from cheese not only as particular species but as strains which can be traced in their distribution throughout the ripening period. The identification of a large number of isolates is, however, limited by the long and tedious processes involved in biochemical tests, which often do not give a fine enough differentiation. Serology, which enables larger numbers of isolates to be identified at one time and with much greater precision, depends largely upon the skill and experience of the specialist worker and involves much preliminary preparation of sera.

It was suggested in a series of papers (Mattick, Cheeseman, Berridge & Bottazzi, 1956; Berridge, Cheeseman, Mattick & Bottazzi, 1957; Cheeseman, Berridge, Mattick & Bottazzi, 1957) that the patterns obtained by paper chromatography of the extractable free amino compounds of micro-organisms could be used for identification. Most of this work was carried out with members of the genus *Lactobacillus*, so it was considered that an experiment investigating the lactobacilli of Cheddar cheese would be a useful test of chromatography as a routine investigational technique.

The lactobacilli present in Cheddar cheese during ripening were studied by Naylor & Sharpe (1958*a*, *b*). They isolated twenty-one serological types, of which only a few were present in large numbers, from a single cheese during the ripening period. The basic flora consisted of two unclassified homofermentative serological types which were partially replaced by a *L. brevis* type in the later stages of ripening. From the evidence produced by these authors it was considered that a cheese after

about four months' ripening would probably produce the greatest variety of lactobacilli.

In the present investigation two Cheddar cheeses 4 months old from different batches were examined.

METHODS

Two 40 lb cheeses, A (2Q 3/12) and B (2Q 4/12) were made from raw milk by the normal Cheddar cheesemaking process in the Institute's experimental dairy. The cheeses were ripened at 55 °F. Two different pairs of starters were used for each cheese, NCDO 504 and 509 for A and 712 and 799 for B. The samples for examination were taken by removing three cores from each cheese when the cheeses were 4 months old. The top $1\frac{1}{2}$ in. of each core was discarded and the rest cut into slices. A 9 g sample from the three cores was taken and weighed into a sterile flask, 80 ml of 2% sodium citrate solution at 50 °C was added and the sample homogenized in a blender for 3 min. The homogenate was allowed to stand 15 min before plating.

Isolation and identification of lactobacilli

Dilutions of the homogenate from each of the two samples were plated in duplicate in acetate agar (AcA) made according to the method of Mabbitt & Zielinska (1956). The plates were incubated at 30 °C for 5 days and 55 colonies were picked from suitable plates, for each of the cheeses. The colonies were transferred into acetate broth. The cultures were purified by streaking on to AcA and picking off single colonies into tomato dextrose broth (T.D.B.). Cultures were Gram stained and tested for catalase production by the addition of 0·5 ml of 20 vol. H_2O_2 to 1·5 ml of a broth culture. All cultures picked from AcA were Gram positive, catalase negative bacilli or coccobacilli.

The biochemical tests were carried out at 30 °C. The method of Gibson & Abdel-Malek (1945) was used in testing for the production of gas from glucose, the inoculum being the deposit from a centrifuged 10 ml T.D.B. culture. For growth in the presence of Teepol, 0·05 ml of vigorously growing culture in T.D.B. was inoculated into T.D.B. containing 0·1% Teepol. Observations for growth were made after 2 and 7 days. The methods described by Briggs (1953) were used for testing growth at 15 and 45 °C. Harvesting of the cells, extraction and chromatography were carried out according to the methods described by Mattick *et al.* (1956).

RESULTS

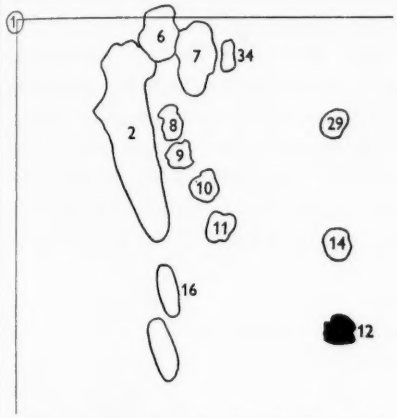
Isolation and preliminary identification

The total counts for the lactobacilli isolated on AcA were 3×10^8/g from cheese A and 8×10^8/g from cheese B.

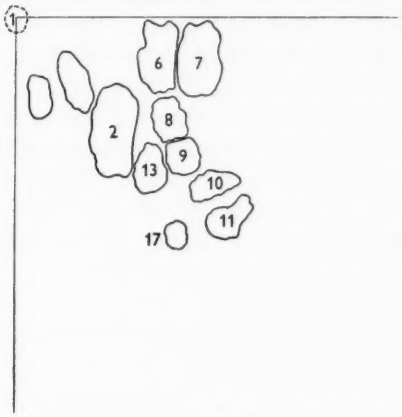
All the 109 isolates (one isolate from B failed to grow) were considered to be lactobacilli as they were Gram positive, catalase negative bacilli or coccobacilli. They grew at 15 °C but not at 45 °C, and in 0·1% Teepol. Most of the strains (96%) did not produce gas from glucose and were therefore considered to be low-temperature homofermentative lactobacilli. Five strains produced gas from glucose, and showed the typical reactions of *L. brevis*.

Chromatographic identification

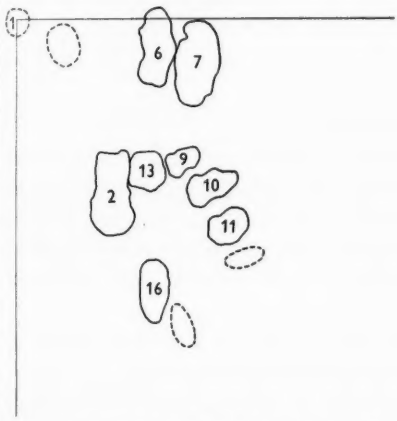
The chromatogram patterns obtained were sorted into four main groups. Chromatogram group 1 consisted of five strains giving patterns similar to those previously described for the low optimum temperature heterofermentative lactobacilli (Cheeseman & Silva, 1959). Of the chromatograms of these strains, one differed from the others in showing the presence of aminobutyric acid and having no colour at the



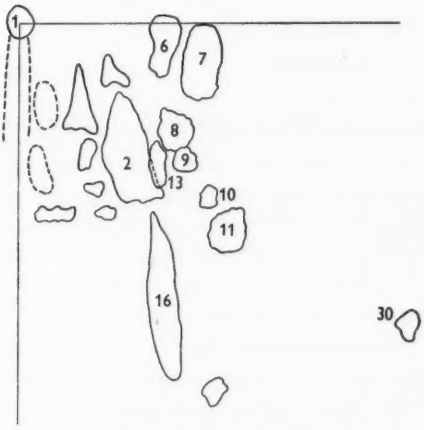
Chromatogram group 1. *Lactobacillus brevis* strains. The aminobutyric acid spot present in group 1(*b*) is shown solid.



Chromatogram group 2. Indeterminate streptobacteria strains. The presence of serine separates these from group 3.



Chromatogram group 3. Indeterminate streptobacteria strains. Serine absent.



Chromatogram group 4. *Lactobacillus plantarum* strains.

Fig. 1. Outline drawings of chromatograms representative of each of the chromatogram groups. Broken line indicates faint spots. The origin in each chromatogram is in the upper left-hand corner. The solvents used were, in the horizontal direction, *n*-butanol–water–acetic acid, 4:5:1 (v/v) and in the vertical direction, *m*-cresol–phenol, 2:1 (v/v) saturated with borate buffer pH 8·3. The presumed identities of the spots are as follows: 1, origin; 2, lysine region; 6, aspartic acid; 7, glutamic acid; 8, serine; 9, glycine; 10, threonine; 11, alanine; 12, aminobutyric acid; 13, asparagine; 14, tyrosine; 16, arginine; 17, glutamine; 30, valine or methionine; 29, 34, and unnumbered spots have not been identified.

origin. An unusual feature of these patterns was the presence of spot 34 (see Fig. 1, group 1) which had only previously been reported for one *L. brevis* strain, MRSB (see Cheeseman & Silva, 1959). The absence of glutamine and spot 15 (unidentified spot running near proline), is contrary to the evidence previously reported for low optimum temperature heterofermentative lactobacilli strains (88 % positive for one or both of these two features, Cheeseman & Silva, 1959). This may suggest that these strains tend to be intermediate between the low and high optimum temperature groups of the heterofermentative lactobacilli. Two of the *L. brevis* strains AA 25 and T 21 previously isolated from cheese are also intermediates. The bulk of the isolates gave patterns intermediate between those of *L. casei* and *L. plantarum* (Cheeseman *et al.* 1957). They were sorted into two groups according to the presence or absence of serine, chromatogram group 2 serine present and chromatogram group 3 serine absent. Seven strains gave patterns which were similar to those previously obtained for *L. plantarum* (Cheeseman *et al.* 1957), and formed chromatogram group 4. Examples of the chromatograms from groups 1–4 are shown in Fig. 1. The distribution of lactobacilli by chromatogram group in each of the two cheeses examined is shown in Table 1.

Table 1. *Distribution of lactobacilli in two Cheddar cheeses*

		A, no. of strains	B, no. of strains
Patterns similar to those of the low optimum temperature group of heterofermentative lactobacilli	Group 1(*a*)	3	1
	(*b*)	0	1
Patterns similar to those of intermediates of casei-plantarum group	Group 2	41	28
	Group 3	10	18
Patterns similar to those of *L. plantarum*	Group 4	1	6
Total strains		55	54

Comparison of chromatogram and biochemical grouping

Several strains conforming to the different chromatogram patterns were selected at random for examination by biochemical methods, two strains from chromatogram group 1, one of which, 4/2*a*, showed aminobutyric acid in its pattern, two strains from group 3 and two from group 4. Also ten strains from group 2 were examined to enable more precise information to be obtained as to any possible variation within a chromatogram group.

The biochemical characteristics of nine of these strains are shown in Table 2. The biochemical grouping was in agreement with chromatogram groups.

The two strains of heterofermentative lactobacilli chromatogram group 1 were both *L. brevis*. They differed slightly from each other in that one fermented mannitol whilst the other did not.

The ten strains of group 2, the characteristics of only three of which are shown here, were all similar. Fermentation of rhamnose, salicin and sorbitol was variable, being usually slight or absent. These strains differed from *L. casei* in failing to hydrolyse aesculin or to ferment salicin strongly, and in growing in 0·1 % Teepol. They also differed from *L. plantarum* in failing to ferment melibiose and raffinose. These strains also must therefore be regarded as indeterminate streptobacteria.

The two strains in group 3, which also differ slightly from each other, are also indeterminate streptobacteria, more closely related to *L. casei* than *L. plantarum*, in failing to ferment cellobiose, showing variable fermentation of salicin, and hydrolysing aesculin weakly or not at all.

Both strains in group 4 were typical strains of *L. plantarum*.

No serological tests were done on any of these isolates.

Table 2. *Biochemical characteristics of strains of lactobacilli isolated from cheese*

Chromatogram group ...	1		2			3		4	
Cheese ...	A	B	A	A	B	B	B	A	B
Strain ...	3/3a	4/2a	3/7/1	3/34/2	4/51/1	4/33	4/48/1	3/5	4/24
Gas from glucose	+	+	−	−	−	−	−	−	−
Hydrolysis of arginine	+	+	.	.	.	.	.	.	.
Growth in 0·1 % Teepol	+	±	+	+	+	±	±	+	+
Growth in 6·0 % NaCl	.	.	−	−	−	+	−	+	+
Hydrolysis of aesculin	−	−	−	−	−	±	−	+	+
Fermentation of:									
Amygdalin	.	.	+	+	+	−	−	+	+
Arabinose	+	+	−	−	−	±	−	+	+
Cellobiose	.	.	+	+	+	−	−	+	+
Dextrin	.	.	−	−	−	−	−	+	+
Glycerol	.	.	−	−	−	+	+	−	−
Mannitol	+	−	+	+	+	+	+	+	+
Melezitose	−	−	+	+	+	−	−	+	+
Melibiose	−	−	−	−	−	−	−	+	+
Raffinose	−	−	−	−	−	−	−	+	+
Rhamnose	−	−	−	−	+	−	−	±	−
Salicin	−	−	−	−	±	+	−	+	+
Sorbitol	.	.	−	−	±	+	+	−	−
Sucrose	−	−	−	−	−	±	±	+	+
Trehalose	−	−	−	−	−	−	−	+	+
Xylose	+	+	−	−	−	−	−	−	−
Identified as	*L. brevis*		Indeterminate streptobacteria			Indeterminate streptobacteria		*L. plantarum*	

All strains grew at 15 °C and not at 45 °C. All the homofermentative strains fermented glucose, fructose, lactose, maltose and mannose. Neither of the heterofermentative strains was tested for fermentation of these five sugars. +, positive growth or fermentation; −, negative growth or fermentation; ±, slight growth or fermentation; ·, not tested.

DISCUSSION

The object of this work was to show whether the technique of chromatography could be used routinely as an adjunct to accepted bacteriological techniques. To assess the possible usefulness of the technique it is necessary to consider three main points. Is the amount of information obtained from a chromatogram pattern sufficient to enable strains to be recognized? Is the technique reliable and the degree of replication good? Is there an advantage in the amount of time, labour and equipment required in relation to the amount of information obtained when compared with other methods?

With regard to the first point, comparison of the biochemical results in Table 2 with the chromatogram pattern groupings and reference to previous papers (e.g.

Mattick *et al.* 1956; Cheeseman *et al.* 1957; Cheeseman & Silva, 1959) shows that in many instances strain differentiation is possible. As to the second point, careful control of the technique using standardized conditions will give good replication of chromatogram patterns obtained from the same strain on different occasions. However, it must be noted that faint or weak spots on a chromatogram will tend to be variable and should not be used as diagnostic features. For the third point, the technique compares favourably with the other methods used. When carrying out the identification of many isolates, the number which can be dealt with by biochemical tests is limited. The serological methods are quicker but need much preliminary work in the preparation of sera. Examination of the cultures took 4 weeks, including the duplication of nearly 50 %, i.e. a total of 160 chromatograms. The cultures were isolated and the preliminary bacteriological tests carried out during the first week, and chromatograms were subsequently prepared from actively growing cultures at the rate of twelve a day. This number is about optimum for one worker per day.

It is not suggested that the chromatographic pattern alone can be used to identify species and strains of lactobacilli, although it is often possible to place the culture into one of the three subdivisions of the lactobacilli—the streptobacteria, thermobacteria and the betabacteria—and even into the appropriate species. In the work discussed here it was first considered necessary to confirm that all the cultures investigated were lactobacilli. Growth at 15 and 45 °C, gas from glucose and growth in 0·1 % Teepol were used as a further indication as to the identity of the cultures.

Confirmation of the chromatogram grouping is given by the fermentation reactions of the examples shown in Table 2. The *L. brevis* strain having aminobutyric acid on the chromatogram pattern differs biochemically from the other *L. brevis* strains in not fermenting mannitol. However, as all typical strains of *L. plantarum* are known to ferment mannitol and also have aminobutyric acid on their chromatogram patterns, it appears doubtful whether there is any direct connexion between mannitol fermentation and accumulation of aminobutyric acid. The chromatogram groups 2 and 3 are biochemically shown to be indeterminate streptobacteria. Group 2, with serine present, ferments amygdalin, cellobiose, and melezitose while group 3, with serine absent, does not ferment these three sugars but ferments glycerol and sorbitol. The fermentation reactions of the two strains from group 4 are distinct from the other strains examined and are typical of *L. plantarum*. This confirms the identification made from the chromatogram pattern.

The distribution of the groups of lactobacilli is shown in Table 1. It is not proposed to place any significance upon this distribution as the sample taken was small, and Naylor & Sharpe (1958*a*, *b*) have already pointed out that the microflora of Cheddar cheese tends to occur irregularly in small localized areas throughout the cheese. Nevertheless, it is suggested that these results indicate that the technique can be usefully employed in problems requiring the rapid identification of strains of micro-organisms.

The author is grateful to Dr A. T. R. Mattick and Dr N. J. Berridge for their interest and encouragement, and to Dr M. E. Sharpe who kindly carried out the biochemical characterizations given in Table 2.

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Production of acetone and acetaldehyde by lactic streptococci

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Summary. Seven strains of *Streptococcus cremoris*, four strains of *Str. lactis*, and one strain of *Str. diacetilactis* were examined for their ability to produce aldehydes and methyl ketones when grown in skim-milk. All strains were found to produce acetaldehyde and seven also produced acetone. No other aldehydes or methyl ketones were detected in any of the cultures.

A method for the quantitative estimation of mixtures of aldehydes and methyl ketones by means of paper chromatography of their 2,4-dinitrophenylhydrazones is described.

It is well recognized that lactic acid is not the sole product of the metabolism of 'homofermentative' lactic streptococci. Small quantities of fatty acids, alcohols, carbonyl compounds (particularly acetoin and diacetyl), and carbon dioxide are known to be formed.

Production of carbonyl compounds other than acetoin and diacetyl has been reported. Acetaldehyde production was reported in skim-milk cultures of *Str. lactis* by Virtanen & Nikkila (1944), and in cultures of *Str. lactis* var. *maltigenes* and other *Str. lactis* strains by Zuraw & Morgan (1952). Jackson & Morgan (1954) found that, in addition to acetaldehyde, *Str. lactis* var. *maltigenes* produced 2- and 3-methylbutanal in skim-milk cultures, while other strains of *Str. lactis* studied did not.

McLeod & Morgan (1958) demonstrated that of several strains of *Str. lactis* and *Str. cremoris*, only *Str. lactis* var. *maltigenes* and one other strain of *Str. lactis* (to a lesser extent) were able to degrade a variety of amino acids to the corresponding aldehydes. The production of acetone and other methyl ketones has not been reported.

In experiments not yet reported the author has found that 1-day-old Cheddar cheese contains a range of methyl ketones and acetaldehyde in concentrations often higher than can be accounted for by the small amounts of these compounds present in the cheese milk. These findings suggested that some of the compounds might be produced by the starter organisms. The results of a study of the production of aldehydes and methyl ketones in skim-milk by strains of *Str. cremoris*, *Str. lactis* and *Str. diacetilactis* are reported in this paper.

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EXPERIMENTAL METHODS

Cultures

All cultures were grown in autoclaved skim-milk (115 °C for 20 min) at 30 °C for 16 h. The inoculum was 2%.

To prevent foaming during the subsequent distillations, 50 ppm. of Dow-Corning Antifoam A, in the form of Antifoam Emulsion AF, was added to the bulk milk before distribution into flasks and autoclaving. Previous experiments had shown that addition of Antifoam to the medium had no effect on either the growth or carbonyl production of the cultures.

Estimation of aldehydes and methyl ketones

Monty (1958) described a method for the estimation of aliphatic carbonyl compounds as their 2,4-dinitrophenyl (2,4-DNP) hydrazone derivatives employing column chromatography and absorption spectrophotometry. A method for the estimation of keto-acids, using similar techniques, was devised by Cavallini & Frontali (1954).

The method described below is applicable to the estimation of aldehydes and methyl ketones, in microgramme quantities, and is similar in principle to the methods referred to above.

A 50 ml quantity of the sample to be analysed was pipetted into a 100 ml flask, and steam distilled. The distillate was led via a suitable adapter directly into 2 ml of 2,4-DNP hydrazine reagent (1 g/l. in 2N-HCl), contained in a 25 ml graduated, glass-stoppered test-tube. When 23 ml of distillate had been collected, any precipitate adhering to the adapter was washed into the test-tube with exactly 2 ml of chloroform, followed by a further 2 ml of 2,4-DNP hydrazine reagent. The tube was then stoppered, and held at 45 °C in a water-bath for 2 h. A reagent blank, containing chloroform (2 ml), 2,4-DNP hydrazine reagent (4 ml), and distilled water (23 ml), was prepared and heated in the same manner.

After removal from the water-bath and cooling to room temperature, each tube was shaken vigorously for 30 sec. A portion of the solvent layer was removed, and 0·05 ml of this extract applied to a Whatman no. 1 chromatogram paper. The paper was then developed, using the method of Lynne, Steele & Staple (1956). After development, the spots were identified from their $R_{\text{but.}}$ values (movement relative to 2,4-DNP hydrazone of butan-2-one) and absorption spectra in ethanolic and ethanolic KOH solutions (Walker & Harvey, 1959). In the present study, where only a limited number of compounds was present, $R_{\text{but.}}$ values provided adequate means of identification for routine analyses.

The spots were cut out, care being taken to remove the same area of paper with each spot, and eluted with 5 ml of ethanol. The corresponding spots from the reagent blank were similarly cut out and eluted. After allowing 15 min for elution, the optical density ($D_{\text{max.}}$) of each eluate was measured at its wavelength of maximum absorption, using a Beckman Model DU Spectrophotometer, with the corresponding reagent blank eluate as a solvent blank.

Using the values for molecular extinction coefficients of 2,4-DNP hydrazones, given by Braude & Jones (1945), the concentration of the parent carbonyl in the milk culture was then calculated from the observed value of $D_{\text{max.}}$.

Accuracy of the method

Expression of the error of the method is complicated by the finding that neither standard deviation (s) nor coefficient of variation (s/mean) remains constant as the mean increases. The mean is actually proportional to s^2, and as a result the fiducial limits are not symmetrical about the mean, as shown in Table 1.

Table 1. *5 % fiducial limits for four observations*

Acetone		Acetaldehyde	
Mean, mg/l.	5 % fiducial limits	Mean, mg/l.	5 % fiducial limits
0·33	0·24–0·44	0·45	0·38–0·53
0·74	0·53–0·97	1·63	1·39–1·89
1·56	1·24–1·91	5·98	5·64–6·32

It was thought desirable to compare the accuracy of the method with the accuracy of the alkaline salicylaldehyde method, a recognized assay method for acetone (Neish, 1952). The standard deviations calculated from results obtained by the two methods proved to be of the same order, which may be taken to indicate that the method is not any less accurate than established micro-methods.

Design of experiments

Autoclaved skim-milk contains variable quantities of both acetone (0·3–0·8 mg/l.), and acetaldehyde (0·1–0·6 mg/l.). To determine whether these compounds were actually produced in skim-milk cultures, it was necessary to compare concentrations in the cultures and in uninoculated samples of the same autoclaved milk, after incubation of both cultures and controls at the same temperature. The differences in acetaldehyde and acetone concentration between cultures and controls were in many cases so large as to leave no doubt that the compounds in question were produced in the culture as a result of the growth of the organism. In other experiments, however, where the differences were small in relation to the initial concentration in the medium, statistical treatment of the results was necessary to confirm whether the differences were significant. For the sake of consistency, therefore, all results were subjected to an analysis of variance.

In each experiment twelve distillations were carried out, four on control milks and four on each of the two cultures under investigation. This gave sufficient data for the analysis of variance to yield conclusive results. For further confirmation each experiment was repeated at a later date.

RESULTS

Seven strains of *Str. cremoris* and four of *Str. lactis* were examined for production of carbonyl compounds other than acetoin and diacetyl. All strains were found to produce acetaldehyde, and seven produced acetone. No other aldehydes or methyl ketones were produced. A strain of *Str. diacetilactis* was studied and found to produce acetaldehyde, but not acetone. In fact the acetone concentration in milk

inoculated with this organism was reduced to zero, indicating that acetone may be utilized by *Str. diacetilactis*.

A summary of the results is presented in Table 2. The figures given indicate the variation in acetone and acetaldehyde production between different experiments on the same organism, and do not take into account the error of the analytical method.

Table 2. *Production of acetaldehyde and acetone by lactic streptococci, in autoclaved skim-milk at* 30 °C

Organism		Acetaldehyde, mg/l.	Acetone, mg/l.
Species	Strain		
Str. lactis	ML_3	1·0–2·5	Nil
	ML_8	0·4–2·0	0·2–0·5
	ML_2	0·4–1·0	0·3–0·7
	C_{10}	2·0–4·5	0·1–0·4
Str. cremoris	HP	3·0–5·0	0·2–1·0
	K	5·0–9·0	0·5–1·0
	ML_1	2·0–3·0	0·1–0·5
	R_1	1·0–3·5	Nil
	R_6	0·5–2·0	0·3–0·6
	KH	4·5–6·5	Nil
	E_8	0·5–2·5	Nil
Str. diacetilactis	DRC_1	11·0–13·0	Nil

Analyses of variance confirmed production of acetaldehyde at 0·1% significance level in all cases, and production of acetone at 1·0% significance level.

Determination of taste threshold concentrations of acetaldehyde and acetone

To determine whether the amounts of acetaldehyde and acetone produced by lactic streptococci were sufficient to influence the flavour of milk and milk products, solutions of these compounds in skim-milk were made up covering the range of concentrations found in the cultures studied.

The solutions were presented to a panel of six, to determine the least detectable concentration within the range, and to find whether significant differences in taste resulted from addition of quantities of acetaldehyde and acetone similar to those amounts produced in the cultures tested.

Concentrations of the solutions tested were: acetaldehyde 0, 0·5, 1, 3, 5, 7 ppm.; acetone 0, 0·5, 1, 1·5, 2 ppm. Results are shown in Table 3.

Table 3. *Taste threshold concentrations of acetaldehyde and acetone in skim-milk*

Compound	Least concentration	Least concentration difference detectable
Acetaldehyde	0·4 ppm.	0·5–1 ppm.
Acetone	0·5–1 ppm.	1 ppm.

The sensitivity to differences in concentration tended to decrease with increase in concentration.

The quantities of acetaldehyde produced by cultures of streptococci (approx. 0·5–10 ppm.) could therefore produce a significant effect on flavour and aroma of a milk culture. Quantities of acetone produced are generally below 1 ppm., and are unlikely to have any great effect on flavour.

DISCUSSION

The production of acetaldehyde and acetone by a variety of strains of *Str. lactis* and *Str. cremoris* grown in autoclaved skim-milk has been demonstrated in this investigation. The production of acetone appears to be confined to certain individual strains within each species.

Acetaldehyde production by *Str. lactis* has been previously reported, and the finding that all strains of *Str. cremoris* and *Str. diacetilactis* studied also produce this compound suggests that this may be a general property of the lactic streptococci. No other aldehydes were detected in any of the cultures. Jackson & Morgan (1954) and McLeod & Morgan (1958) reported that *Str. lactis* var. *maltigenes* possessed the ability to convert amino acids to the corresponding aldehydes, by means of a transamination reaction followed by the decarboxylation of the alpha-keto acids thus formed. The skim-milk medium used in the present investigation contained traces of free amino acids (Block, 1951); furthermore, *Str. lactis* has been shown to possess appreciable proteolytic activity, even in 24 h cultures (Morgan & Nelson, 1951). The fact that no higher aldehydes were found in the cultures of *Str. lactis* and *Str. cremoris* studied seems therefore to indicate that the ability to degrade amino acids to aldehydes by the above route is confined almost entirely to *Str. lactis* var. *maltigenes* and is not a general property of the group.

The accumulation of acetaldehyde can be explained as resulting from the decarboxylation of pyruvic acid, which can be formed by transamination of alanine, or, much more likely in the present case, as a result of glucose metabolism.

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Strontium-90 from fallout in the diet and milk of a dairy herd*

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SUMMARY. The diet and milk from a herd of dairy cows were analysed regularly for ^{90}Sr and calcium. The 'observed ratio' (the ratio of ^{90}Sr to calcium in the milk divided by the ratio of ^{90}Sr to calcium in the diet) was found to be 0·10 against ^{90}Sr in favour of calcium, which corresponds well with the results of other workers. The amount of ^{90}Sr secreted into the milk was 1·2% of the total ingested. Similar values were obtained using stable strontium data.

Soil samples from different depths and herbage were taken from three areas with different cultivation histories and analysed for ^{90}Sr and calcium. The effect of ploughing on the vertical distribution of ^{90}Sr in soil and on the ^{90}Sr levels in vegetation was examined.

In recent years the radioactive content of agricultural produce has been increased by contamination with fallout from the testing of atomic weapons. With the smaller (kiloton) weapons, the radioactive debris is completely deposited within a few weeks and is confined to the latitude of the test site. With larger (megaton) weapons, however, the fission products are carried into the stratosphere and, as a result of the stable conditions in this region, deposition continues over several years and is distributed over at least the whole of the hemisphere in which the explosion occurred. According to Stewart, Crooks, Osmond & Fisher (1957), most of the fallout in the United Kingdom is stratospheric in origin and because of the storage time in the stratosphere, its radioactivity is due mainly to fission products with long half-lives. In estimating the potential hazard to man from the various constituents of fallout, it is obvious that those which are absorbed from the gastro-intestinal tract and retained in the body for long periods are the most important. ^{90}Sr falls into this category and, in addition, it is more readily absorbed by plants from contaminated soil than are other fission products and is secreted into the milk of cows grazing contaminated pasture. For these reasons the metabolism of strontium, both in plants and animals, has been studied intensively in the past few years. Surveys of ^{90}Sr in soil, pasture, vegetables, milk and human bone have also been undertaken in a number of countries to establish the current levels of contamination and to

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enable some predictions to be made of trends (Bryant, Chamberlain, Morgan & Spicer, 1957).

In Western countries, milk and milk products constitute the principal source of man's dietary calcium. As strontium moves similarly to calcium in food chains, milk might also be expected to be the principal source of ^{90}Sr. This is in fact so, although it has been shown that the cow discriminates against strontium in favour of calcium, so that the strontium/calcium ratio in the milk is less than that in the diet. The reason for this has been discussed by Bryant, Chamberlain, Spicer & Webb (1958).

A useful concept in studying the comparative movement of calcium and strontium in biological systems is that of Comar, Russell & Wasserman (1957) who proposed the strontium-calcium observed ratio (*OR*) as a measure of the over-all discrimination that occurs in the movement of the two elements from one phase to another. For the movement from diet to milk the *OR* may be defined:

$$OR_{\text{milk-diet}} = \frac{\text{Sr/Ca in milk}}{\text{Sr/Ca in diet}}.$$

Comar *et al.* (1957) have summarized the values obtained for this ratio by various workers using double tracer experiments, and also the results of ^{90}Sr surveys. The values ranged from 0·08 to 0·16. The experiment to be described here was planned to measure this discrimination under natural conditions using a large dairy herd, so that differences in metabolism which are known to exist between individual cows would be taken account of. The dairy herd at the Sonning Farm of the University of Reading was used for this experiment. Samples of the various dietary constituents were taken and analysed at 3-weekly intervals and representative milk samples were also analysed. The mean ^{90}Sr/calcium ratio of the diet was compared with that for the milk and a value for the discrimination obtained.

On the same farm, samples of soil were taken from various depths in three selected areas to find how cultivation had affected the vertical distribution of ^{90}Sr. Grass samples taken from the same sites showed how the distribution in the soil affected the levels in the vegetation.

MATERIALS AND METHODS

Diet of the dairy herd

The Sonning dairy herd contained some 75 cows during the course of the experiment, about two-thirds of these being Friesian and the remainder Shorthorn. The diet of the herd was similar to that of many herds in the country in that hay, silage, kale, straw and concentrates were used during the winter, whilst grazing provided the bulk of the summer requirement. Table 1 summarizes the feeding, average milk yield and size of the herd during the course of the experiment.

The grazings were grass clover swards of varying age and composition. For a short period, which included sample 15, lucerne was grazed. A small amount of the silage used in the early months of 1957 came from an arable crop of cereal and vetches, but all the remainder was made from either grass or lucerne. The swards were cut for silage at intervals varying from 4 months for the first cut to about 6 weeks for

subsequent cuts during the period May to September. The material was ensiled in a pit, clamp or tower silo, usually with the addition of molasses. The hay was made from grass cut during June and July, baled and stored under cover. The straw was derived from cereals (chiefly barley) grown on the farm. All silage, hay and straw used during the investigation were less than 1 year old.

Table 1. *Rations fed, milk yield and herd size*

Sample no.	Dates	Average per cow per day, lb			Average no. of cows
		Bulky foods	Concentrates	Milk yield	
1–3	28.xi.56–8.i.57	Kale 80, straw 6	4·9	18·9	72
4–6	29.i.57–20.iii.57	Kale 40, silage 30–40, straw 8	7·1	23·5	69
7–14	23.iv.57–1.x.57	Grazing	1·0	28·7	78
15	19.xi.57	Grazed lucerne 40, silage 50, hay 10	2·0	24·4	78
16–19	10.xii.57–11.ii.58	Kale 40–60, silage 60–80, hay 10	4·4	24·8	73

The kale used during both winters was sown in rows 22 in. apart during the previous April to June. It was left unthinned and was grazed behind an electric fence. The herd was allowed access to a fresh strip at intervals of from 1 to 3 days.

The concentrate ration was mixed on the farm, its composition varying according to the price and availability of feedingstuffs. It consisted of 35–70% of cereals, together with groundnut cake, fishmeal and other minor ingredients. A mineral mixture containing chalk and bone flour was also added.

During the winter of 1957–58, two concentrate rations were used. Mixture A was fed to cows yielding up to 3 gal of milk per day, whilst mixture B was fed to the remainder. The chief difference between the two mixtures was that in mixture B, approximately half the wheat and barley were replaced by flaked maize.

Sampling at the farm

Since the aim of the investigation was to study the ^{90}Sr content of the diet and milk of the dairy herd as a whole, samples were taken on a herd basis and no attempt was made to obtain information relating to individual cows.

While the herd was on winter feed, the amounts of hay, silage, straw, kale and concentrates fed were estimated as part of the normal farm practice. Of this data, the kale figures are probably the least accurate because of the present difficulties of assessing quantities eaten by grazing animals.

It was also difficult to measure accurately the amount of herbage eaten by the herd when at grass during the summer. It is undoubtedly true that the type and length of sward, weather conditions and other factors can influence herbage consumption, but it was not possible to conduct continual grazing trials to measure the intake at each time of sampling. Instead, a single trial was made over a period of 4 days on two different swards. Ten samples were collected before each grazing and a similar number were collected from adjacent sites after grazing. The results obtained showed

the average daily consumption of herbage dry matter to be about 24 lb/cow. Although the night grazing period was nearly twice the length of the day period, consumption was equally divided between the two. Throughout the investigation herbage consumption was assumed to conform to these results. Any inaccuracy entailed by the assumption of a standard daily herbage intake will have increased the errors of calculating the ^{90}Sr intake but will have had little effect on the calculation of the ^{90}Sr/calcium ratio of the diet, since the only other component of the diet during the summer was a small quantity of concentrates, representing less than 5 % of the total dry-matter intake.

Samples of the foods were collected in the afternoon on a day when no change in the feeding had taken place during the preceding 3 days. With the hay, straw, silage and concentrates, 2–3 kg were taken from the material to be fed to the herd that night or next morning. A similar weight of kale was taken at random from the area to be grazed by the cows next morning. Since the lower parts of the kale stem were left largely uneaten, they were discarded from the sample.

When the cows were at grazing, separate pastures were usually grazed by day and by night. From each sward, three samples, each of 1 m^2, were cut with shears about 1 in. from the ground on the area next to be grazed. Although controlled strip-grazing was the normal practice, the herbage so collected did not fully represent the herbage consumed by the herd, because no allowance could be made for any selectivity in grazing habit.

The milk sample consisted of bulked evening and morning milk collected 24 and 36 h after the food sampling.

Radiochemical analysis

All samples of diet were weighed as received and again after drying at 110 °C for 12 h. The dried samples were ashed at 700–800 °C and the ash weighed. After thorough mixing, a sample of ash (4 g) was taken for analysis. The ash together with strontium carrier (50 mg) was evaporated almost to dryness in a platinum dish with 30 ml each of perchloric acid (72 %, w/w) and hydrofluoric acid (40 %, w/w). After the addition of more perchloric acid (30 ml) evaporation was continued to remove traces of fluoride. The residue was dissolved as completely as possible in water with the addition of a small amount of nitric acid and any residue remaining was filtered off. After diluting the filtrate, strontium and calcium were co-precipitated as the oxalates by the addition of an excess of oxalic acid and enough ammonia to bring the pH to a value of 4. The oxalate precipitate was filtered into a tared sintered-glass Gooch crucible and decomposed to the carbonate by heating at 500 °C. Subtraction of the weight of strontium carbonate (84·2 mg) equivalent to the added carrier from that of the precipitate gave the weight of calcium carbonate and hence the calcium content of the ash sample. The calcium was removed from the strontium by the fuming nitric acid procedure, details of which have been given by Bryant, Chamberlain, Morgan and Spicer (1956), who also describe the subsequent purification steps. Briefly these consist of barium chromate and ferric hydroxide scavenging precipitations, which remove any radioactive barium and radium isotopes which may be present. Finally, the purified strontium was stored in solution with yttrium carrier (10 mg) for not less than 18 days to allow the ^{90}Y daughter to equilibrate with the

^{90}Sr. After this period had elapsed, the yttrium was separated as the hydroxide, converted to the oxalate, mounted, and counted with an end-window Geiger-counter on at least three occasions to check the 64 h half-life of the ^{90}Y. The activity of this isotope at the time of separation from the strontium, after correction for the recovery of strontium carrier and counter efficiency, gave the ^{90}Sr activity of the original sample. The counter was calibrated with yttrium oxalate sources prepared from a standard ^{90}Sr-^{90}Y solution supplied by the Isotope Division of the Atomic Energy Research Establishment. Replicate determinations of ^{90}Sr in a sample of ash showed that the reproducibility was about $\pm 5\%$.

Initially, representative samples of liquid milk were spray-dried and subsequently ashed at 700–800 °C. Later, however, they were charred by dripping the liquid into a hot silica beaker, the contents of which were finally ashed. The analysis of ash for calcium and ^{90}Sr followed exactly the methods described by Bryant *et al.* (1956). Determinations of ^{89}Sr were made on the various milk samples. To do this, the purified strontium was precipitated as the carbonate after separation of the yttrium carrier, mounted, and counted after the ^{90}Y had re-equilibrated. Subtraction of the count due to the ^{90}Sr-^{90}Y from the total gave that of the ^{89}Sr. Corrections were applied for carrier recovery, counter efficiency, self absorption and radioactive decay, to determine the ^{89}Sr activity at the time of sampling.

The soil samples were extracted with 6 N-HCl and the calcium and ^{90}Sr content of the extract determined. This gives the total rather than the 'exchangeable' soil calcium. It has been shown by Bryant *et al.* (1957) that there is little difference between the ^{90}Sr content of soils measured by this procedure and by extraction with neutral ammonium acetate solution.

RESULTS AND DISCUSSION

^{90}Sr *in diet and milk*

The results of the calcium and ^{90}Sr determinations on the various constituents of the diet are given in Table 2. The values in the last column show which materials contribute most ^{90}Sr per unit weight of calcium to the diet. Grass samples collected during the summer of 1957 had the highest values, and kale collected the previous winter had the lowest of the arable crops. The reasons for the difference in activity will be discussed later. The concentrated feedingstuff had the lowest value of all, but this is certainly due to the addition of mineral calcium in its preparation.

Table 2. ^{90}Sr *and calcium in constituents of diet*

Material	No. of samples	Sampling period	Calcium, g/kg dry wt.	^{90}Sr, $\mu\mu$c/g calcium	
				Range	Mean
Grass	16	iv.57–x.57	7·75	20–110	48
Hay	4	xi.57–ii.58	5·71	31–40	36
Silage	3	i.57–iii.57	7·24	19–20	20
Silage	4	xi.57–ii.58	6·31	25–41	33
Straw	6	xi.56–iii.57	3·22	33–48	41
Lucerne	2	xi.57	12·67	29–36	33
Kale	6	xi.56–iii.57	10·59	6–21	11·5
Kale	3	xii.57–ii.58	9·89	21–26	23
Concentrates	20	xi.56–ii.58	5·97	3–15	6·6

Another source of calcium and ^{90}Sr is drinking water. For water supplies in this area the calcium content is about 0·1 g/l. Assuming that a cow drinks 15 gal of water daily, the calcium intake will be about 7 g per day, which is significant compared with the calcium from other sources. Some figures for the ^{90}Sr concentration in river and well water have been given recently by Stewart, Crooks, Osmond, Owers & Healy (1959). The ^{90}Sr content of water from the River Thames at the time of the experiment was about 0·2 $\mu\mu$c/l. The concentration in tap water could be no greater than this, and the ^{90}Sr content of well water is likely to be very much less due to adsorption of activity on the soil. As the herd was supplied with either mains or well water during the experiment, the contribution of ^{90}Sr to the diet from this source may be neglected.

Taking into account the calcium in the drinking water, the mean calcium and ^{90}Sr contents of the diets grouped as in Table 1 have been calculated, and in Table 3 are compared with the amounts secreted into the milk. The mean values for the ^{90}Sr/calcium ratio in the diet and milk are also included, together with the $OR_{milk-diet}$ values. The mean value and standard deviation for the observed ratios is 0·10 ± 0·03, which corresponds well with the results of other investigations. The values obtained for the *OR* during the summer months appeared to be somewhat lower than in winter, which may simply mean that representative samples of pasture were not obtained. On average, 1·2 % of the ingested ^{90}Sr was secreted into the milk. During the course of the experiment the amount of ^{90}Sr secreted into the milk dropped considerably, and this may be related to the increase in the amount of calcium ingested over the same period.

Table 3. *Daily calcium and* ^{90}Sr *contents of diet and corresponding milk samples (per cow)*

	Diet			Milk				
		^{90}Sr			^{90}Sr		^{90}Sr secreted into milk, %	$OR_{milk-diet}$
Periods	Calcium, g	$\mu\mu$c	$\mu\mu$c/g calcium	Calcium, g	$\mu\mu$c	$\mu\mu$c/g calcium		
1–3	83·0	700	8·4	10·4	13·9	1·3	1·48	0·16
4–6	94·7	1810	19·1	11·7	21·0	1·8	1·16	0·10
7–14	93·5	3780	40·4	14·3	40·3	2·8	1·13	0·07
15	125·0	3310	26·5	13·0	41·6	3·2	1·26	0·11
16–19	134·1	3400	25·4	13·0	30·0	2·3	0·95	0·09

The radiostrontium activities of the various milk samples are shown in Fig. 1. The low ^{90}Sr levels in the first three samples (1·2–1·4 $\mu\mu$c/g calcium) are explained by the fact that the diet at this time consisted almost entirely of kale which had a low ^{90}Sr/calcium ratio. The addition of silage to the diet with a reduction in the amount of kale increased the milk activity to 1·7–2·0 $\mu\mu$c/g calcium, and immediately the herd moved out to pasture in April the value increased to 3·3. In the winter of 1957–58, the activity of milk did not drop to the extremely low values of the previous year. No doubt this was due in part to increasing levels of ^{90}Sr in the environment.

The activity found in the milk is low compared with samples from other parts of Britain. The eight samples analysed in the summer of 1957 averaged 2·8 $\mu\mu$c ^{90}Sr/g calcium, whilst samples from Somerset in the same year averaged 5·1 (Bryant, Morgan & Spicer, 1958).

Fig. 1 also shows the ^{89}Sr levels. This isotope has a relatively short half-life (51 days) compared with ^{90}Sr and also occurs in fallout. The low levels in winter milk show how radioactive decay had reduced the ^{89}Sr content of stored foodstuffs, and the transition from winter feed to pasture is clearly marked by a sudden increase in the ^{89}Sr level. The abnormally high value of 87 $\mu\mu c/g$ calcium in milk sampled on 2 October 1957 followed a sharp rise observed by Stewart, Osmond, Crooks, Fisher & Owers (1959) in the specific activity of this isotope in rain water collected during the previous month.

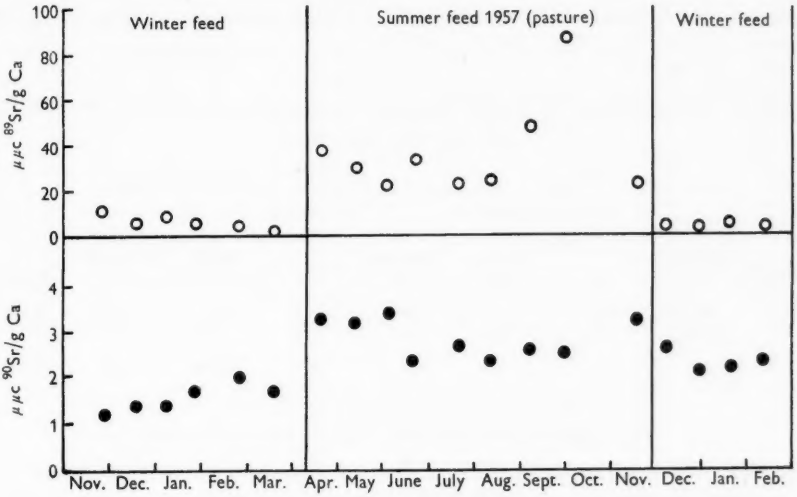
Winter feed
Summer feed 1957 (pasture)
Winter feed
μμc 89Sr/g Ca
μμc 90Sr/g Ca
Nov. Dec. Jan. Feb. Mar. Apr. May June July Aug. Sept. Oct. Nov. Dec. Jan. Feb.

Fig. 1. ^{89}Sr and ^{90}Sr activities in milk.

Table 4. ^{90}Sr *and stable strontium in constituents of diet and milk*

Period	Material	^{90}Sr, $\mu\mu c$/g calcium	Stable strontium, mg/g calcium	^{90}Sr/stable strontium, $\mu\mu c$/mg
12	Pasture (day)	39	2·25	17·3
	Pasture (night)	72	2·25	32·0
	Concentrates	11	2·75	4·0
	Milk	2·4	0·24	10·0
17	Kale leaf	28	2·20	12·7
	Kale stalk	25	4·15	6·0
	Silage	41	2·35	17·4
	Hay	40	2·30	17·4
	Concentrates (A)	6	1·45	4·1
	Concentrates (B)	5	1·20	4·2
	Milk	2·1	0·25	8·4

Stable strontium in diet and milk

As ^{90}Sr and stable strontium are chemically indistinguishable it would be expected that the discrimination against either with respect to calcium would be the same, provided that they are in the same chemical form in the diet. In two sampling periods, each item of the diet and the ash from the corresponding milk samples were analysed

by flame photometry for stable strontium. The results are given in Table 4, which also shows the ^{90}Sr/stable strontium ratios. The highest values for this ratio are found in pasture and other grass crops, indicating that some of the ^{90}Sr has been taken up directly without first equilibrating with the stable strontium in the soil.

In Table 5, the mean stable strontium/calcium ratio of the diet in each of these two periods is compared with that for the corresponding milk sample. The values for the $OR_{milk-diet}$ and for the fraction of the stable strontium intake which is secreted into milk are similar to the mean values obtained from the ^{90}Sr data. The calcium and stable strontium in the cows' drinking water have again been taken into account, assuming a daily intake of 7 g calcium and a stable strontium content of 2·4 mg/g calcium.

Table 5. *Daily calcium and stable strontium contents of diet and corresponding milk samples (per cow)*

	Diet			Milk				
		Stable strontium			Stable strontium		Stable strontium secreted into milk	
Period	Calcium, g	mg	mg/g calcium	Calcium, g	mg	mg/g calcium	%	$OR_{milk-diet}$
12	88·9	202·2	2·27	15·4	3·71	0·24	1·83	0·11
17	136·0	311·3	2·29	13·1	3·28	0·25	1·05	0·11
						Mean	1·44	0·11

The effect of ploughing on ^{90}Sr in soil and grass

One field at Sonning consists of three areas (A, B and C) which were ploughed and sown to grass at different times. The soil types in these areas are very similar, all being light sandy loams with 75–80 % of coarse and fine sand and 10–15 % of clay. Area A was last ploughed in April 1953, at which time the world deposition of ^{90}Sr was very slight, so that for present purposes A can be regarded as permanent grass. Area B was ploughed annually from 1953, the last occasion being in January 1956. Area C was similarly treated and in addition was ploughed in April 1957.

In July 1957 soil samples were taken from three sites on each of the three areas described. Each sample was divided into three parts, the 0–2 in., 2–6 in. and 6–12 in. horizons. The samples from the same horizon in each area were then bulked and thoroughly mixed before being analysed for ^{90}Sr and total calcium. Table 6 gives the results obtained.

The data show that whilst the total amount of ^{90}Sr in the three areas varies by little more than 5 %, there is a marked difference in the vertical distribution. In A, nearly 80 % of the ^{90}Sr is in the top 2 in. of soil, whilst in B and C the figures are 57 % and 31 % respectively. These differences indicate the relative immobility of strontium in the soil. Miller & Reitemeier (1957) have shown that even under heavy leaching, stroutium is slow to move down the soil profile. Thus in area A the absence of ploughing has resulted in a concentration of ^{90}Sr in the top horizon. In B and C regular ploughing has caused a redistribution of the ^{90}Sr. The pattern of redistribution will no doubt be affected by the method of ploughing and the condition of the soil at the time, since both these factors can affect the degree to which the soil is inverted.

Herbage samples were taken at the same sites from which the soil samples were obtained and analysed for ^{90}Sr and calcium. The results are shown in Table 7. The herbage samples differed considerably in their calcium content and this is probably due to plant differences. Area A carried less clover than B, and C contained a high proportion of weeds, notably Redshank (*Polygonum persicaria*).

Table 6. ^{90}Sr *and total calcium in soil samples*

Area	Last ploughed	Horizon, in.	Total calcium, g/kg dry weight	^{90}Sr		
				$\mu\mu c/m^2$	%	$\mu\mu c/g$ calcium
A	April 1953	0–2	2·2	4550	79	30
		2–6	2·3	750	13	2·5
		6–12	3·3	450	8	0·7
				5750		
B	January 1956	0–2	2·5	3100	57	16
		2–6	2·5	1600	29	4·4
		6–12	1·4	750	14	1·5
				5450		
C	April 1957	0–2	2·5	1700	31	9·6
		2–6	3·3	2350	42	6·0
		6–12	3·0	1500	27	2·3
				5550		

Table 7. ^{90}Sr *and calcium in grass samples*

Field	Mean dry weight, kg/m^2	Mean calcium content, g/kg dry weight	^{90}Sr			
			$\mu\mu c$/kg dry weight		$\mu\mu c$/g calcium	
			Samples	Mean	Samples	Mean
A	0·158	4·64	606, 511, 476	531	120, 124, 100	115
B	0·149	8·69	323, 415, 299	346	38, 46, 35	40
C	0·100	13·09	255, 312, 216	261	23, 27, 13	20

Entry of ^{90}Sr into plants takes place by root absorption from the soil, and for grass with its large leaf area it is likely that uptake by leaves is also important. In addition, Russell (1958) has suggested that ^{90}Sr retained in the root-mat, which often develops under permanent grass swards, will be more accessible to plants than if it had become incorporated in the soil. Of the sites examined in the present study only area A had any root-mat, and the relatively high ^{90}Sr content of the herbage samples from this area is probably due partly to the concentration of ^{90}Sr in the top two inches of soil and partly to absorption from the mat.

In Fig. 2, the $^{89}Sr/^{90}Sr$ ratios in milk samples, collected while the cows were at grass, are compared with the ratios measured in monthly rain-water samples for the same period by Stewart, Osmond, Crooks, Fisher & Owers (1959). The high values for this ratio in milk, and particularly the way in which they follow those in rain, indicate that much of the radiostrontium in milk and therefore in grass must be of recent origin. This 'fresh' radiostrontium is probably taken up directly by the leaves, stems and surface-roots of the grass, rather than from the soil. This conclusion is

supported by the ^{90}Sr/stable strontium ratios in Table 4, which are higher for grass herbage than for other crops, showing that some of the ^{90}Sr has been taken up directly and has not equilibrated with the stable strontium in the soil.

The data presented suggest that regular ploughing and re-seeding of grassland (i.e. ley farming), can reduce the ^{90}Sr/calcium ratio in herbage. Direct absorption is partly reduced by preventing the formation of a root-mat, and since most British grasses are shallow rooting, ploughing will place the surface accumulation of fallout below the zone from which nutrients are mainly absorbed. This conclusion is in agreement with data published by Russell & Milbourn (1957). The average ^{90}Sr and calcium contents of grass from this farm (see Table 2) correspond in general with those found in B, indicating that the husbandry practised has reduced the levels below those to be expected from permanent grass. Since grass and grass products

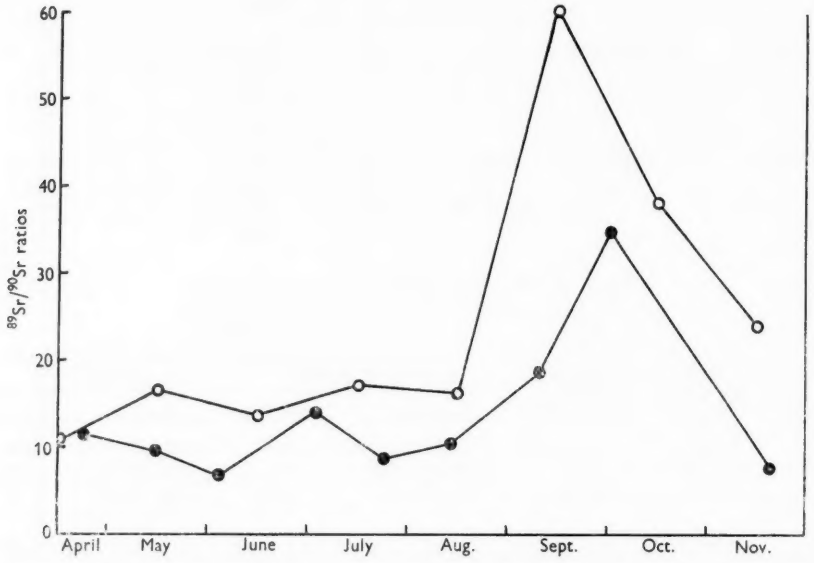

Fig. 2. A comparison of $^{89}Sr/^{90}Sr$ ratios in rain water and in milk during the summer of 1957. ○, Ratio in rain (at mid month); ●, ratio in milk (at time of sampling).

are the main diet of most dairy herds, reduction in the levels in herbage can be expected to reduce the levels in milk. Ultimately, of course, ploughing will bring to the surface some of the active material previously buried so that soil inversion may well become less effective in reducing uptake of ^{90}Sr while fallout continues at a steady or increasing rate. After a short period of heavy fallout, levels in herbage could be effectively reduced by deep ploughing, followed for some years by shallow rotary cultivation.

The extent to which these comments apply to other crops depends in part on their rooting habits. In the present study, kale gave lower ^{90}Sr to calcium ratios than grass. This may be due to the fact that its roots penetrate any surface accumulation of ^{90}Sr and tap a larger soil reservoir. Alternatively, it could be due to the lower

leaf area per unit of ground covered and lower plant population, resulting in a decreased capacity for aerial or stem-base absorption. The importance of these various factors must be determined in detailed experiments and cannot be assessed from studies of the present type.

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Chemical changes in dried skim-milk during storage

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Summary. Dried skim-milk stored at 55 °C in air at 70 % relative humidity has been found to contain lactulose, galactose, tagatose, glyceraldehyde and maltol; and formic, acetic and glycollic acids. These compounds are not present, or are present only in trace quantities, in fresh dried skim-milk. It is suggested that while the protein–sugar reaction is responsible for most of the browning of the powder, many of the compounds found are formed by degradation of lactose catalysed by the free basic amino groups of the casein. The compounds so formed probably then react with amino groups in a Maillard reaction and thus contribute to the browning of the milk powder.

Dried skim-milk, when stored under warm humid conditions, deteriorates because of undesirable physical and chemical changes which lead eventually to development of a brown colour and a loss of nutritive value. It has been assumed that this browning is caused by interaction between the lactose and the amino compounds present in milk. Lea (1948) and Lea & Hannan (1949) demonstrated that in the 'dry' state glucose reacts with the free amino groups of casein in a 1:1 ratio. Lea & Hannan (1950) later showed that in the earlier stages of the interaction the amino groups involved are predominantly the ϵ-amino groups of lysine. The complex so formed browns readily (Lea, Hannan & Rhodes, 1951) under suitable conditions of temperature, pH and humidity.

Other workers have determined the nature of the substances formed during the browning of heated (usually autoclaved) liquid milk. Among the compounds found are formic (Gould, 1945*a*), acetic (Keeney, Patton & Josephson, 1950), and lactic (Gould, 1945*b*) acids, furfuryl alcohol (Patton & Josephson, 1949), maltol (Patton, 1950*a*), acetol (Keeney *et al.* 1950), methyl glyoxal (Keeney *et al.* 1950), 5-hydroxymethyl furfural (Patton, 1950*b*) and lactulose, galactose and tagatose (Adachi & Nakanishi, 1958). Many of these substances identified in heated milk have also been reported as alkaline degradation products of lactose. This suggests that the browning of heated milk involves a degradation of lactose which is base-catalysed, probably by the free amino groups of casein and in particular by the ϵ-amino groups of the lysine units in the casein molecule.

Conditions in skim-milk powder during storage are very different from those obtaining in heated fluid milk. Confirmation is needed, therefore, that the basic

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mechanism of browning is the same in dry milk systems as in fluid milk. The investigations described in this paper cover an examination of dried skim-milk which had been stored under warm humid conditions.

EXPERIMENTAL AND RESULTS

Chromatography. Unless otherwise stated, in the following experiments sheet-paper partition chromatography was done by the descending method on Whatman no. 1 paper using *n*-butanol—ethanol—water (40:11:19 by vol.) as the mobile phase.

Storage of dried skim-milk. Dried skim-milk (2 kg) was stored at 55 °C in air at 70 % relative humidity for 21 days, during which time it became brown in colour and formed a solid cake. This cake was ground to a fine powder in a mortar.

Aqueous extraction of brown powder. A portion (250 g) was extracted with distilled water (750 ml) for 3 h at 60 °C. The solids were separated by filtration and re-extracted twice with distilled water (250 ml). The extracts were combined and concentrated.

Isolation of compounds from the aqueous extract. The concentrate deposited crystals on standing. These crystals were separated by centrifugation and were identified as lactose by paper chromatography and the formation from them of lactosazone.

Column chromatography. Organic acids and bases were removed by slowly adding a portion (50 ml) of the mother liquor (75 ml) from the above separation, to a column (1·5 cm × 15 cm) of Amberlite resin IR 120(H), the eluate from which dripped on to a column (1·5 cm × 15 cm) of Amberlite resin IR 45(OH). The columns were then exhaustively washed with water. The neutral eluate and washings were concentrated under vacuum, and after a further crop of lactose crystals had been removed the concentrate (12 ml) was transferred to a cellulose powder column (3·5 cm × 33 cm) and fractionated by partition chromatography using butanol half saturated with water at room temperature as the mobile phase. The effluent was collected portion-wise and after examination on paper chromatograms was grouped into fractions which were concentrated.

Fraction 1. This (0·03 g) contained a compound with chromatographic properties similar to maltol (R_F 0·79). More conclusive evidence for the presence of maltol in brown skim-milk powder is given in the later section 'Ethyl acetate extraction of brown powder'.

Fractions 2–4. There were present in these fractions small quantities of compounds with R_F values of 0·38, 0·32 and 0·25, respectively. They were not investigated further.

Fraction 5. This syrup (0·2 g) contained a compound which had the R_F value (0·17), spray reactions, and especially the red colour with resorcinol-hydrochloric acid spray, characteristic of a ketohexose. It was indistinguishable from tagatose but distinct from the other ketohexoses on paper chromatograms using three solvent systems: (*a*) *n*-butanol—ethanol—water (40:11:19 by vol.), (*b*) ethyl acetate—acetic acid—water (9:2:2 by vol.) and (*c*) phenol—water (100:39 by wt.). Its *R* values in these solvents were (*a*) R_F 0·17, (*b*) R_{fructose} 1·1, and (*c*) R_F 0·43.

Fraction 6. This on chromatographic analysis was found to contain tagatose (R_F 0·17) and a very small quantity of a compound R_F 0·14.

Fraction 7. This syrup (0·8 g) contained a sugar indistinguishable on paper chromatograms from D-galactose (R_F 0·09). The methylphenylhydrazone had m.p. 188 °C undepressed on admixture with genuine galactose methylphenylhydrazone m.p. 189 °C.

Fraction 8. This (0·24 g) on paper chromatograms moved to R_F 0·013 and gave a red colour with the resorcinol-hydrochloric acid spray characteristic of a sugar containing a ketohexose moiety. Thus the sugar was a glycosyl ketose. A portion (0·04 g) was purified by chromatography on sheets of Whatman no. 3MM paper. The band containing the glycosyl ketose was cut out, extracted with methanol and the extract concentrated. The extract gave with aqueous phenylhydrazine acetate an osazone which, after recrystallization from aqueous ethanol, had the characteristic crystal form of lactosazone and had m.p. 199 °C undepressed on admixture with genuine lactosazone. The glycosyl ketose was thus identified as lactulose.

Hydrolysis of lactulose. The sugar (0·1 g) was dissolved in 2 % oxalic acid (2 ml) and the solution heated in a sealed hydrolysis tube at 100 °C for 1 h. The mixture was then neutralized with Amberlite resin IR 45(OH). Paper chromatographic analysis showed the presence of unchanged lactulose (R_F 0·013), a compound (0·02 g) with the same R_F (0·09) and colour reactions as galactose and also a compound with the same R_F (0·11) and colour reactions as the ketohexose fructose. The identification of galactose and fructose was confirmed by further paper chromatography using as solvent ethyl acetate—acetic acid—formic acid—water (18:3:1:4 by vol.).

Table 1. *Non-volatile neutral compounds identified in dried skim-milk stored at 55 °C in air at 70 % relative humidity*

Compound	Fraction	R_F	Identification
Maltol (3-hydroxy-2-methyl-γ-pyrone)	1	0·79	Parent compound*†, ultra-violet spectra, colour reactions
Tagatose	5	0·17	Paper chromatography
Galactose	7	0·09	Methylphenylhydrazone*†
Lactulose	8	0·013	Lactosazone*†‡, ketose reactions, hydrolysis to galactose and fructose

* M.p. † Mixed m.p. ‡ Crystalline form.

Non-volatile organic acids. The aqueous extract from browned powder (250 g) was slowly added to a column of Amberlite resin IR 45(OH) and the column then washed exhaustively with distilled water (eluate I). Eluate I was retained to be examined for carbonyl compounds. Hydrochloric acid (0·2 N) was then slowly added to the column until the eluate gave a strong positive test for chloride (eluate II). Eluate II was neutralized with silver carbonate and filtered. Silver ions were removed from the filtrate with hydrogen sulphide and the solution was again filtered. The filtrate was extracted continuously with ether. The ether was removed and the residue dissolved in water and examined by paper chromatography. The solvents used were (*a*) 1-pentanol saturated with 5 M-formic acid (Buch, Montgomery & Porter, 1952), and (*b*) propanol—ammonia—water (6:3:1 by vol.) (Isherwood & Hanes, 1953). After development the papers were hung in a draught of air for 4 h to remove the last traces of solvent. Papers developed in solvent (*a*) were then sprayed with a 0·04 % ethanolic solution of bromophenol blue which had been adjusted to pH 7. The results (Table 2)

show that three of the seven acids present, citric, glycollic, and lactic, could be identified by paper chromatography. The presence of these acids was confirmed by the colour reactions (Feigl, 1947) given by aqueous solutions of acids which had been separated as bands on sheets of Whatman no. 3MM paper.

Carbonyl compounds. Eluate I (50 ml) was steam distilled, the distillate being led directly into 25 ml of 2,4-DNP reagent (2 g of 2,4-dinitrophenylhydrazine in 1 l. of 2N-HCl). The reaction mixture was extracted with carbonyl-free chloroform (10 ml) and 0·03 ml aliquots were chromatographed by the methods of Lynn, Steele & Staple (1956) and Huelin (1952) (Table 3). The absorption spectra of the separated carbonyl

Table 2. *Non-volatile organic acids present in dried skim-milk stored at* 55 °C *in air at* 70 % *relative humidity*

Acid	R_F in 1-pentanol—formic acid	R_F in propanol—NH_4OH—H_2O (6:3:1)	Powder stored at 55 °C	Fresh powder	Remarks
Citric	0·22	Streaks	S	M	Confirmed by spot test
	0·32	0·29	M	—	
Glycollic	0·39	0·41	M	W	Confirmed
	0·52	0·22	M	W	
Lactic	0·60	0·58	S	S	Confirmed
	0·73	0·71	M	—	
	0·83	0·82	M	—	

S = strong spot; M = medium-strength spot; W = weak spot.

Table 3. *Properties of* 2,4-DNP *compounds of volatile neutral carbonyl compounds present in dried skim-milk stored at* 55 °C *in air at* 70 % *relative humidity*

	Parent carbonyl compound			
	Unknown	Glyceraldehyde	Acetaldehyde	Acetone
$R_{butanone}$ (Lynn, Steele & Staple, 1956)*	0·21	0·32	0·43	0·69
R_F (Huelin, 1952, descending)	0·00	0·24	0·39	0·52
Absorption maxima (mμ). Solvent:				
Ethanol	375	352	357·5	360
Ethanolic KOH				
(*a*) Major	465	430	430	430
(*b*) Subsidiary	—	—	490 (inflexion)	525
Heptane	360	337	342	345
Concentration relative to glyceraldehyde in fresh powder				
(*a*) Fresh powder	0·4	1	2	1·8
(*b*) 55° storage	6	9	15	4

* $R_{butanone}$ = rate of movement relative to that of the 2,4-dinitrophenylhydrazone of butan-2-one.

2,4-DNP compounds in ethanol and ethanolic KOH were recorded using the techniques of Walker & Harvey (1959). A similar method was used to measure the absorption spectra in heptane solution. The results of these experiments are also shown in Table 2.

DL-Glyceraldehyde for comparison was prepared by dissolving glycerol (10 ml) in bromine water (100 ml) and warming the mixture at 60 °C until the bromine colour disappeared. The resulting solution was steam distilled and the steam distillate was led directly into 2,4-DNP reagent (5 ml). The chloroform extract of this reaction mixture was examined by paper chromatography and the absorption spectra of the resolved 2,4-DNP compounds were measured by the methods described above. On papers developed by the method of Lynn *et al.* (1956) two spots were found, one at R_{butanone} 0·33 and the other on the base-line. The spot at R_{butanone} 0·33 had the same absorption characteristics as the spot R_{butanone} 0·32 found in the 2,4-DNP compounds from stored milk powder, and gave the reddish brown colour with alcoholic KOH expected of a mono-2,4-DNP. The spot on the base-line gave the strong violet colour with alcoholic KOH characteristic of a bis-2,4-DNP.

Ether extraction of browned powder. The brown powder (2 kg) was extracted by stirring with ether (3 l.). The extract was separated by filtration and the powder re-extracted twice with further ether. The combined extracts were concentrated and then dissolved in water.

Ether-soluble organic acids. Paper chromatography of the ether extract by the method of Reid & Lederer (1951) showed the presence of formic, acetic and/or lactic, and butyric acids. The presence of lactic acid was confirmed by paper chromatography using the method of Denison & Phares (1952). Confirmation of the presence of formic, acetic and butyric acids was obtained by gas-phase chromatography of the free fatty acids on a column packed with Celite 545, silicone D.C. 550, behenic acid and *o*-phosphoric acid (10:4:0·4:0·4) as the stationary phase and nitrogen as the mobile phase.

Keto acids. Keto acids present in the ether extract were examined as their 2,4-dinitrophenylhydrazones by paper chromatography using the method of Cavallini, Frontali & Toschi (1949) as modified by Dagley, Fewster & Happold (1952). There was a pink spot on the papers at R_F 0·81 which proved to be maltol carried through the various extractions. There was a yellow spot at R_F 0·47 ($\lambda_{\text{max.}}$ in 10 % Na_2CO_3 and 1·5N-NaOH (1:1) at 4700 Å) which was not identified. For comparison the 2,4-DNP compounds of keto acids known to have similar R_F values were examined on the same paper and it was found that the 2,4-DNP of pyruvic acid had R_F 0·75 and 0·54 ($\lambda_{\text{max.}}$ at 4450 Å), that of laevulinic acid had R_F 0·62 ($\lambda_{\text{max.}}$ at 4300 Å) and that of glyoxylic acid R_F 0·43 ($\lambda_{\text{max.}}$ at 4500 Å).

Ethyl acetate extraction of brown powder

Maltol. Browned powder (1 kg) was extracted continuously with ethyl acetate under reduced pressure controlled so that the temperature of the extract did not rise above 55 °C. The extract was concentrated under vacuum and the concentrate dissolved in distilled water. The solution was extracted with petroleum ether, the extract was discarded and the aqueous solution was then extracted with ethyl acetate. The extract was concentrated and dried to a syrup (9 g) from which needle-like crystals (0·1 g) separated. These were collected on the filter. They had m.p. 158 °C raised to 161·5 °C on recrystallization from aqueous ethanol. The compound had R_F 0·79, turned ferric chloride solution reddish violet, Folin-Ciocalteau reagent a deep blue, and

reduced ammoniacal silver nitrate in the cold. The ultra-violet spectra of the compound in 0·1 N-sulphuric acid (E_M = 8800 at 275 mμ) and in 0·1 N-sodium hydroxide (E_M = 7197 at 317 mμ) were very similar to those reported for maltol (Schenck & Speilman, 1945). On the above evidence the compound has been identified as maltol.

Storage at 37 °C. Dried skim-milk stored at 37° C and 70% r.h. did not brown as rapidly as powder stored at 55 °C, but chromatographic examination of an extract showed that similar chemical changes were taking place.

Examination of fresh dried skim-milk

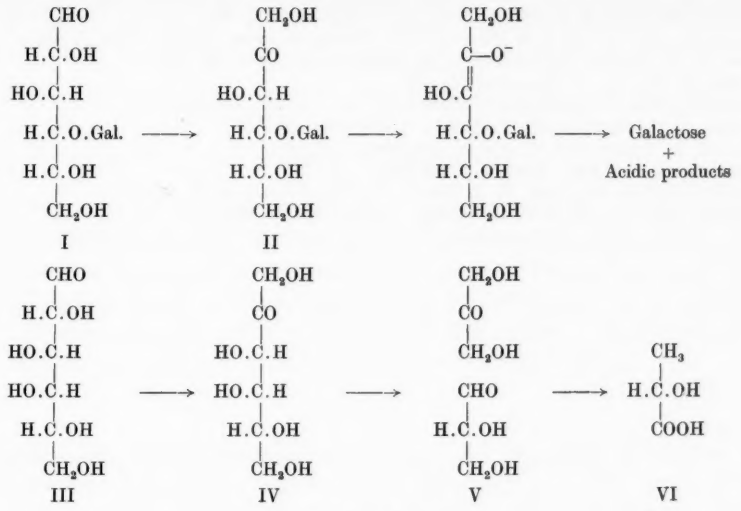
Aqueous extraction. Fresh dried skim-milk (250 g) was reconstituted with distilled water (750 ml). Sulphuric acid (0·2 N) was added to the reconstituted milk and the precipitated casein then removed by filtration. The mother liquor was concentrated under reduced pressure and then examined for reducing sugars by paper chromatography. Only lactose and a trace of galactose were revealed in contrast to the lactose, lactulose, galactose and tagatose in an aqueous extract of similar concentration from browned dried skim-milk. The concentrate was also examined for non-volatile acids and carbonyl compounds. The results are shown in Tables 1 and 2.

Ethyl acetate extraction. Fresh dried skim-milk (500 g) was extracted by stirring with ethyl acetate (2 × 500 ml). The extract was concentrated and dissolved in water (5 ml). Paper chromatographic examination revealed maltol as a very faint spot in contrast to the very strong spot given by an ethyl acetate extract of similar concentration from browned dried skim-milk.

DISCUSSION

Many of the compounds identified as being formed in skim-milk powder during storage are known to be products of the alkaline degradation of lactose. The formation of these compounds has to be explained in the light of the observation of Lea (1948) and Lea & Hannan (1950) that reducing sugar actually combines with casein to form an amino—sugar complex and that in the earliest stages of the interaction, before discoloration develops, this is virtually the only reaction occurring. The isolation of maltol (3-hydroxy-2-methyl-γ-pyrone) is evidence that such an amino—sugar interaction is taking place, since simple alkaline degradation of lactose does not produce maltol whereas degradation with glycine or casein does (Patton, 1950). The decomposition of such an amino—sugar complex could cause most of the browning occurring in skim milk powder but could not lead to the presence of compounds normally associated with the alkaline degradation of lactose. Amino—sugar complexes have been shown to decompose with dilute acids, even dilute acetic acid, yielding not hydrolysis products but the parent amino compound and 5-hydroxymethyl furfural (Gottschalk & Partridge, 1950; Richards, 1956). Under neutral conditions Richards (1956) found no sugar epimers in a glucose—glycine mixture. Even if lactulose (4-β-D-galactosyl-D-fructose) was formed from the amino—sugar complex an explanation would still be needed for its further degradation to galactose and tagatose. It is suggested that the alkaline degradation products of lactose are formed not from an amino—sugar complex but by a degradation of lactose catalysed by the free basic amino groups of casein. Such a degradation of lactose in the milk system would follow a sequence of

reactions similar to that postulated by Corbett & Kenner (1953) for the degradation of lactose with strong alkali, thus:



(Gal. = galactosyl.)

Lactose (I) is pictured as slowly epimerizing to lactulose (II) the fructose moiety of which is degraded so that lactulose gives galactose and acidic products. The galactose (III) so formed is epimerized to tagatose (IV) which can then be fragmented into trioses (V). The trioses can finally be oxidized to acids (VI). Lactose, lactulose, galactose, tagatose and glyceraldehyde, were all identified in the stored skim-milk powder. Although the powder was not examined for saccharinic acids, of the acids identified formic, acetic, lactic and glycollic are known to be formed during the alkaline degradation of sugars. The reactive decomposition products of lactose formed can then react with amino groups in Maillard reactions and so contribute to the browning of the milk powder.

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The use of turbimetric methods for testing homogenized milk

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Summary. A detailed study has been made of spectroturbimetric methods for testing the degree of homogenization of milk, and it has been shown that, provided care is exercised in the design of the turbimeter, the turbidity index at a specified wavelength provides a useful practical measure of the degree of homogenization. This measurement can easily be made with a simple filter turbimeter.

Several turbimetric methods have been proposed for testing the degree of homogenization of homogenized milk (Ashworth, 1949; Lagoni & Merten, 1955; Deackoff & Rees, 1957; Goulden, 1958*a*, *b*; Haugaard & Pettinati, 1959). This paper discusses the factors involved in the design of instruments used for the measurement of turbidity, and attempts to assess the value of turbimetric tests as control methods for the production of homogenized milk.

Theoretical aspects of light scattering

Goulden (1958*c*), has already discussed in detail the factors affecting the turbidity of diluted milk samples. When the dilutions are large enough to avoid effects due to multiple scattering and the protein has been solubilized by the addition of alkali, the turbidity index (T_λ) at any wavelength λ can be expressed by the relationship:

$$T_\lambda = (y/lx) \log (I_0/I) = yd/lx, \quad (1)$$

where y = volume dilution factor; l = optical path length in cm; x = fat content of the undiluted milk in g/100 g; I_0 = intensity of radiation falling on the sample; I = intensity of radiation after passing through the sample and d = optical density or absorbance. As confirmed later by experiment, the optical density is directly proportional to the fat content and inversely proportional to the dilution, in accordance with this formula.

Using the method of Goulden (1958*c*), T_λ values could in principle be calculated theoretically for the differing fat globule size distributions obtained by homogenizing a sample of milk at a series of different pressures. In practice, it was found impossible to make these calculations owing to the experimental difficulty of the accurate sizing of the smaller globules. Despite this, theoretical considerations still enable the qualitative effects of homogenization upon T_λ to be predicted.

EXPERIMENTAL

Turbidity measurements at narrow band-widths were made with a modified Unicam SP.500 spectrophotometer (Goulden, 1958*d*), the main feature of this modified instrument being the increased separation of the sample cell and the detector.

Measurements were made at wide band-widths using a modified Miller colorimeter (Goulden, 1958*d*) and a modified Hilger Biochem Absorptiometer (Goulden, 1958*a*). In the latter instrument, a Megatron type I.R. barrier-layer photocell was used, and the galvanometer shunt removed to obtain correct damping. An $\frac{1}{8}$ in. $\times \frac{1}{16}$ in. aperture stop was fitted into the optical path of the Biochem Absorptiometer at the focus in front of the detector. Both absorptiometers were used with Wratten no. 87 and Kodak NP.587/3 filters, the effective maximum sensitivities of the two filter-detector combinations being close to 0·8 and 0·9μ respectively. (A recent communication from the manufacturers of these filters states that filter NP.587/3 has now been superseded by filter NP.580. This has a similar spectral transmission, but is more stable to light.) Milk and cream samples were warmed to about 40 °C before use to ensure that the fat was in the liquid state.

Fat determinations were made on homogenized milk samples by the Gerber method (British Standard 696), using repeated centrifuging to ensure complete fat separation.

Sterilized homogenized milk samples kindly provided by the Cheshire Sterilized Milk Co. Ltd., were tested by the centrifugal method of Ridgway (1957).

RESULTS

Effects due to alkali addition

Ashworth (1949) and Deackoff & Rees (1957) solubilized the protein by the addition of 5N-ammonium hydroxide solution, allowing the diluted milk samples to stand for 30 min before measurement. Preliminary tests showed that dilute sodium hydroxide solution was as effective as ammonium hydroxide and that measurements could be made immediately after the addition of this reagent. Provided an amount of sodium hydroxide equivalent to a minimum of 1 ml N-NaOH per 1 ml milk had been added, together with the required amount of water for dilution, higher concentrations of alkali did not lead to further reduction in turbidity. Identical results were obtained when milk samples were diluted with approximately 0·004N-NaOH.

Table 1. *Effects due to protein turbidity in diluted milk suspensions*

Wavelength, μ	Percentage of total turbidity due to protein		
	Raw milk	Homogenized milk, 140 kg/cm²	Homogenized milk, 210 kg/cm²
0·6	4	14	16
0·8	2	13	16
1·0	1	12	16
1·2	0·7	11	16

For the measurements with raw milk at longer wavelengths in the previou experiments (Goulden, 1958*d*), it was found unnecessary to add alkali since the

scattering due to the fat globules was very much greater than that due to the protein. With the smaller fat globules in homogenized milk, the relative scattering contribution of the protein was larger and less dependent on wavelength, as illustrated by the results shown in Table 1. These results were calculated from the optical densities of diluted milk samples, before and after the addition of alkali.

When alkali was added to diluted separated milk, a small residual turbidity was still observed. For a dilution of 2 ml separated milk with 2 ml of N-NaOH to 250 ml with water, an optical density of 0·015 was found at 0·6μ, falling to 0·008 at 1·2μ. This difference corresponds to an error of less than +2% in the T' value and is of the same order as the accuracy of the simple filter instrument proposed for routine use (Goulden, 1958*a*).

Effects due to the optical system

Lothian & Chappel (1951) have demonstrated the importance of small angles of acceptance of the light beam at both source and detector. Since the experimental results already obtained with diluted raw milk samples using the modified Unicam SP. 500 spectrophotometer are in good agreement with the calculated values (Goulden, 1958*c*), it would seem that the optical system of this modified instrument is suitable for turbidity measurements with raw milk. As already shown (Lothian & Chappel, 1951), the maximum permissible acceptance angle becomes larger as the globule sizes are reduced or the wavelength increased. This instrument would therefore be expected to give correct turbidity values with homogenized milk samples where the globule sizes are smaller than those in raw milk. Experimental evidence of the suitability of this modified instrument is confirmed by the results shown in Table 2 below.

Table 2. *Optical densities of diluted homogenized milk measured with different cell-detector spacings* (S)

Wavelength, μ	Cell positions (see text) A S = 3 cm	B S = 6 cm	C S = 23·5 cm	D S = 26·5 cm
0·4	0·482	0·800	1·190	1·205
0·6	0·265	0·455	0·580	0·580
0·8	0·191	0·290	0·325	0·331
1·0	0·145	0·197	0·209	0·210
1·2	0·132	0·150	0·152	0·152

When the angle subtended by the illuminated area of the sample at the detector is too large, low optical density values are obtained due to the presence of non-scattered radiation at the detector (Lothian & Chappel, 1951). As this angle is reduced, the measured optical density rises to a limiting value and this is illustrated by the results obtained with the Unicam spectrophotometer and shown in Table 2. Table 2 records the optical densities measured under different conditions for a milk sample homogenized at 140 kg/cm^2 and diluted by making 1 ml milk and 1 ml N-NaOH up to 250 ml with water. Similar sets of results were obtained from milk samples homogenized at 70 kg/cm^2 and at 210 kg/cm^2. In the former the larger globule sizes caused even more pronounced effects to show, whilst the effects with the sample homogenized at 210 kg/cm^2 were less than those with the sample homogenized at 140 kg/cm^2.

In position A, the cells were placed at the end of the cell compartment closest to the photocells. Position B corresponds to that normally used for absorption work with the Unicam spectrophotometer SP. 500, where the cells are placed in the cell compartment at the end farthest from the detector. In positions C and D, the cells were placed in the cell compartment as for positions A and B, the photocell box being fixed behind a $\frac{1}{2}$ in. diameter aperture 10 in. away from the cell compartment. For these differing arrangements, the respective cell-detector separation distances were 3, 6, 23·5 and 26·5 cm.

The results shown in Table 2 confirm that the optical densities approach limiting high values with decrease in the angle subtended by the detector at the sample. This is particularly noticeable at the shorter wavelengths where the ratio of globule size to wavelength is larger.

Position A corresponds to that used normally in the Beckman DU spectrophotometer and also in the Beckman B spectrophotometer when the cells are placed at the end of the cell compartment nearest to the detector. The optical densities obtained under such conditions depend very much upon the actual cell position, and at 0·9μ are only about two-thirds of the values obtained when the detector subtends a small angle at the cell. When the cells are placed as far as possible from the detector in the Beckman B instrument, or unmodified Unicam SP. 500 spectrophotometer, the optical densities at 0·9μ are still about 10 % too low.

These results show how the measured turbidity of the same sample can vary with the instrument used, unless precautions are taken to ensure that a narrow angle is subtended at the detector by the sample.

Effects of dilution

Provided the dilutions are large enough to avoid the effects of multiple scattering, equation (1) shows that the optical density should be inversely proportional to the dilution factor. This relationship is confirmed by the results shown in Fig. 1, which illustrates the changes in optical density obtained from a series of dilutions of a commercial homogenized milk sample, the appropriate amount of alkali being added in each case.

The optical densities of the same diluted milk samples were measured at 0·8 and 0·9μ with the modified SP. 500 spectrophotometer, and also with the modified Biochem Absorptiometer, using both filters.

The linear relationships at 0·8 and 0·9μ confirm the linearity of the optical density—dilution relationship and confirm that the effects of multiple scattering are not apparent at the milk concentrations normally used, $< 0{\cdot}8$ %. The linearity of response of the galvanometer circuit in the absorptiometer is also confirmed by the straight lines obtained with this instrument. Similar results were obtained with the modified Miller colorimeter.

Effects of changes in fat content

In order to check the linearity of the optical density—fat percentage relationship, samples of 'synthetic' homogenized milk were made up by adding known amounts of cream homogenized at 210 kg/cm^2 to separated milk. After being well mixed, the

samples were diluted and examined in the usual way. Table 3 shows the turbidity indices measured at 0·9μ with the Unicam spectrophotometer ($T_{0·9}$).

These results confirm that the T values are independent of the fat content. Under the microscope, a small amount of 'clumping' of the fat globules was observed with the diluted cream samples and it is considered that this factor is probably responsible for the small variations observed in the T values. With normal diluted homogenized milk samples, no clumping effects were observed.

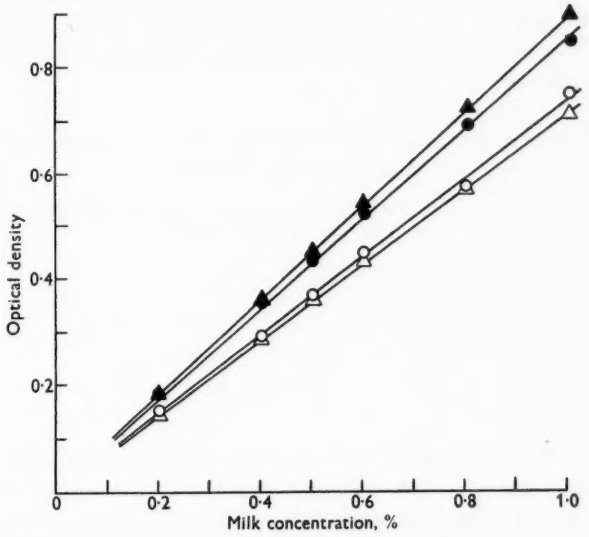

Fig. 1. Effects of dilution upon optical density of homogenized milk. ▲, Unicam at 0·8μ; △, Unicam at 0·9μ; ●, Biochem with filter 87; ○, Biochem with NP 587/3 filter.

Table 3. *Effects of fat content* (x) *on turbidity index measured at* 0·9μ ($T_{0·9}$)

Fat, % (x)	S.N.F. %	Dilution factor	$T_{0·9}$
1·02	8·99	250/2	31·2
1·95	8·91	250/2	30·2
2·92	8·83	250/2	29·7
3·76	8·74	250/1	29·8
4·80	8·66	250/1	31·3
5·76	8·48	250/1	30·6

Effects of wavelength change

Deackoff & Rees (1957) have already shown that the use of longer wavelengths enlarges the differences in turbidities between milk samples homogenized at different pressures. This effect was due partly to the use of too large an acceptance angle at the detector, since they used a Beckman B instrument in which the cell-detector spacing corresponded to position B (Tables 2 and 4). Table 4 shows the percentage difference in turbidity index between portions of the same bulk milk sample examined after homogenization at 210 and 140 kg/cm^2. These differences are shown as functions of both the wavelength and of three different detector positions A, B and D.

From these results, it can be seen that a large cell-detector separation, as in position D, makes the turbimeter more sensitive to changes in homogenization pressure. A correctly designed instrument working at 0·8 or 0·9μ can be seen to have a slightly greater sensitivity than the Beckman B used at 1·02μ in the Deackoff & Rees (1957) method.

In order to find cheap and simple turbimeters for routine use, several filter colorimeters were examined and a suitable instrument for turbidity measurements was found to be the Hilger Biochem Absorptiometer, modified as already described. The long-wavelength limit of this instrument was governed by the sensitivity characteristics of the barrier-layer photocell, and could be extended to about 0·95μ by choice of a suitable photocell.

Table 4. *Effects of wavelength and detector position on sensitivity to change in homogenization pressure*

(% difference in T for milk homogenized at 210 and 140 kg/cm². Detector positions as for Table 2.)

Wavelength, μ	A	B	D
0·4	0	5	13
0·6	4	16	21
0·8	12	21	24
1·0	18	23	25
1·2	20	23	24

Since the effective band-pass of such an instrument is a function of both the filter and detector characteristics, it is suggested that the T values obtained with such an instrument should be related by a suitable calibration factor to those obtained at a definite wavelength with an instrument employing monochromatic radiation. A convenient wavelength for calibration is 0·9μ, since the T values obtained at this wavelength are close to those obtained with the absorptiometer using a Kodak NP.587/3 filter with the Megatron type I.R. photocell.

Fig. 2 shows the calibration data obtained from measurements on a number of homogenized milk samples supplied by different dairies and examined by both instruments. The points fall on a straight line providing a calibration factor (K) of 0·96. T values obtained with the absorptiometer using this filter-detector combination can therefore be converted into T values at 0·9μ ($T_{0·9}$) by multiplying by 0·96. Such $T_{0·9}$ values are independent of instrumental factors and depend solely upon the fat globule size distribution of the milk. The same K value can be derived from the slopes of the appropriate lines in Fig. 1.

Effects of homogenization pressure and comparison with centrifugal tests

A number of experiments were made in which a series of milk samples homogenized at various pressures were examined by both the turbimetric and the centrifugal tests. Fig. 3*a*, *b* illustrates the results of a typical experiment and demonstrates the improved correlation obtained by the turbimetric method. The F value in Fig. 3*b* is used to designate 'homogenization efficiency' as measured by Ridgway's (1957) method.

These results demonstrate the sensitivity of $T_{0\cdot9}$ values to changes in homogenization pressure, especially with the higher pressures where the centrifugal test appears to be less reproducible and where sizing by the microscope becomes increasingly difficult and tedious.

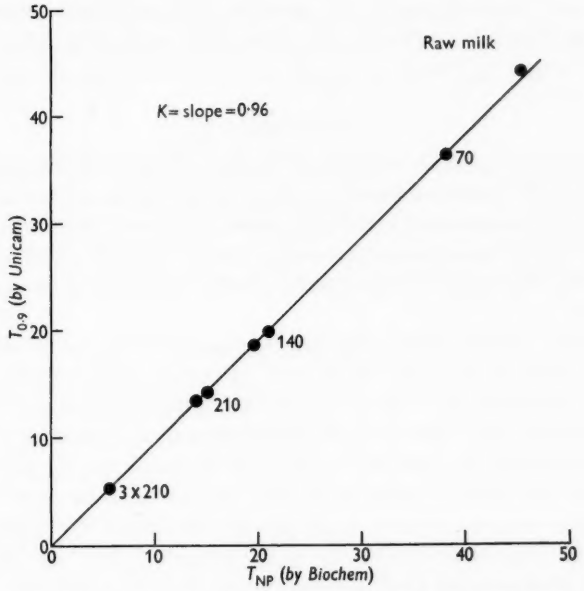

Fig. 2. Calibration data for Biochem Absorptiometer. $T_{0\cdot9}$ is the turbidity index measured at 0·9 μ with the modified Unicam SP. 500 spectrophotometer, T_{NP} is the turbidity index measured with the absorptiometer, using the NP. 587/3 filter. Numbers close to the points indicate homogenization pressure in kg/cm².

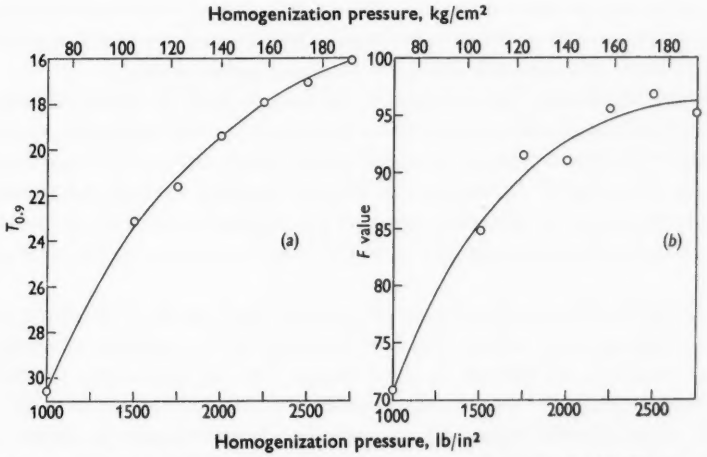

Fig. 3. (*a*) Effect of homogenization pressure on $T_{0\cdot9}$ as measured with Biochem Absorptiometer. (*b*) Effect of homogenization pressure on F values.

Effects of heating and sterilization

Since the $T_{0\cdot9}$ value is independent of protein turbidity, it seemed unlikely that sterilization would affect the turbimetric results, and this was confirmed by the examination of homogenized milk samples before and after sterilization. No change in $T_{0\cdot9}$ values was observed when milk samples were sterilized under commercial conditions, or by in-bottle heating at 92 °C for times up to 1 h.

DISCUSSION

From the foregoing results, it is apparent that provided a correctly designed optical system is used, the T value is a function solely of the degree of homogenization, and therefore of the fat globule size distribution curve. In view of the ease and rapidity of the turbimetric test, it would seem that a turbimetric method might possibly be used in place of the United States Public Health (1947) method as a test for efficiency of homogenization.

A more detailed consideration of the factors relating to fat globule size distribution shows that it is possible to obtain the same T value from milk samples with differing fat globule size distributions. This is due to the difficulty of having to use a single parameter to describe both the average globule size and the spread of globule sizes. It is possible to imagine a mixture of 1μ diameter globules and 3μ diameter globules which will have the same T value as a homogeneous emulsion containing only 2μ diameter globules. It was, in fact, found possible to obtain the same T value for a milk sample homogenized at 140 kg/cm^2 as for a mixture of 1 part of raw milk with 5 parts of milk homogenized at 210 kg/cm^2.

In other words, a milk sample homogenized 'badly' at a very high pressure and containing a few large globules in the presence of many very small ones could have the same T value as a sample homogenized 'well' at a lower pressure, containing only medium-sized globules but no large globules. These larger globules could arise from a defective homogenizer valve or possibly from the coalescence of smaller globules after passing through the homogenizer valve, and in commercial practice it is probably the few large globules still present which are of major importance.

Despite this limitation, the turbimetric method is likely to prove of considerable value in testing homogenizer performance, since any process leading to a reduction in globule sizes will cause a decrease in the T value, irrespective of the way in which the globule sizes are reduced. It may well be found in practice that the fat globule break-up patterns produced by different types of homogenizers are sufficiently similar to enable the degree of homogenization to be indicated by the turbidity index as a single parameter.

The turbimetric test has already found practical application in studying the effects of wear on homogenizer valves, where a decrease in homogenizer efficiency due to valve wear results in an increase in the T value. In this laboratory, the turbimetric test has been extensively used in the preparation and homogenization of synthetic emulsions using different types of homogenizers. Improvements in degree of homogenization, as shown by T values, were always confirmed by examination under the microscope.

Where more detailed information about the fat globule size distribution is required, measurements of T values at several wavelengths, together with examination under the microscope, will be necessary. In the first instance, it would seem that a method based upon centrifugal separation would provide the best method for detecting the presence of a few large globules, but the results shown in Fig. 3 suggest that the results of the turbimetric method are more reproducible.

Homogenized milk samples in current production by a number of commercial dairies have been examined and all were found to have $T_{0\cdot9}$ values in the range 15 to 20.

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A note on the availability of the calcium of milk submitted to ultra-high-temperature sterilization with or without subsequent in-bottle sterilization

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Summary. The effect of ultra-high-temperature (U.H.T.) sterilization and of subsequent in-bottle sterilization on the availability of milk calcium has been studied in experiments with rats. The heated milks and the raw milk from which they were prepared were freeze-dried and added to a diet low in Ca so that the resulting diets contained some 0·11 % Ca and 0·42 % P.

Ca retention by groups of six litter-mate male weanling rats was 98·4, 98·4 and 98·5 % respectively from diets containing raw milk, U.H.T.-treated milk and milk treated by U.H.T. followed by in-bottle sterilization. It is clear that these processes did not affect the availability of milk Ca.

It has been shown, in experiments with rats, that the availability of milk Ca is not affected by pasteurization (Henry & Kon, 1937) or by drying (Shields, Fairbanks, Berryman & Mitchell, 1940). Steggerda & Mitchell (1941) have confirmed the latter finding in man. So far as we are aware no experiments have been done to determine the effect on the availability of milk Ca of more severe heat treatment, such as sterilization, that is known to reduce the nutritive value of milk proteins (see Henry, 1957).

The development of the U.H.T. process for improving the keeping quality of milk led to tests at this Institute on the effect of such treatment on the nutritive value of milk. In the absence of a satisfactory aseptic bottling technique it was necessary for the U.H.T. process to be followed by in-bottle sterilization. Results have already been reported for the effect of these treatments on the vitamins (Chapman *et al.* 1957) and proteins (Henry & Porter, 1959) of milk; those for the availability of Ca are now reported.

EXPERIMENTAL

Milk was sterilized by the U.H.T. process as described by Burton (1958). Half of this milk was further sterilized in bottles in an atmosphere of steam at 110 °C for 30 min. These two samples, together with one sample of the raw milk from which they were prepared, were freeze-dried and stored at −15 °C until required. Table 1 gives the composition and analysis of the experimental diets. Disodium hydrogen phosphate was added as a source of phosphorus so that the diets contained sufficient of it to ensure maximum retention of the suboptimal amount of Ca (see Henry & Kon, 1939). The milks supplied some 90 % of the dietary Ca. Six litters of three male rats aged

25–28 days were used, litter-mates being allocated by lot to the three diets. The two types of metabolic cages used, together with details of the collection and analysis of the excreta for Ca, have been described in detail by Henry & Kon (1937). Excreta were discarded for the first 4 days and then collected for 23 days. P was determined by the molybdivanadate method (Kitson & Mellon, 1944; Hanson, 1950). Some urine samples, when treated with the molybdivanadate reagent, developed a red colour that interfered with the determination. The interference was overcome by evaporating a suitable portion of urine to dryness and ashing with magnesium nitrate solution (Association of Official Agricultural Chemists, 1955). The residue was moistened with water, dissolved in the least quantity of hot 20 % (v/v) nitric acid, filtered and made up to a suitable volume.

Table 1. *Percentage composition of the diets and their content of* Ca, P *and moisture*

	Diet		
Component	Raw milk	U.H.T. milk	U.H.T. milk + in-bottle sterilization
Freeze-dried milk	11·8	12·1	12·2
Egg-white	13·5	13·5	13·5
Margarine fat	7·0	7·0	7·0
Sugar	8·0	8·0	8·0
Ca- and P-free salts*	2·5	2·5	2·5
$Na_2HPO_4.12H_2O$	3·5	3·5	3·5
Maize starch	53·7	53·4	53·3
Vitamin mixture†	0·05	0·05	0·05
Ca‡	0·1127	0·1133	0·1108
P‡	0·4192	0·4103	0·4218
Moisture	9·43	10·24	10·10

* Salt mixture of de Loureiro (1931) with calcium phosphate omitted.

† Inositol 4·4 g, nicotinic acid 1·0 g, Ca-pantothenate 2·0 g, *p*-aminobenzoic acid 1·5 g, aneurin hydrochloride 0·6 g, riboflavin 0·6 g, pyridoxin hydrochloride 0·16 g.

‡ Mean values for two batches of each diet; in calculating the intakes of Ca and P allowance was made for the slight differences in the Ca and P content of the two batches of diet.

RESULTS AND DISCUSSION

The results, given in Table 2, show that 98 % of the Ca was retained from the three milks, a figure identical with that previously found for spray-dried milk under similar experimental conditions (Henry & Kon, 1939). It may be concluded, therefore, that neither the U.H.T. treatment of milk nor the subsequent in-bottle sterilization of it affects the availability of milk Ca. P retention was similar from the three diets, amounting to some 25 % of the ingested P. In our earlier work with dried milk (Henry & Kon, 1939) some 50 % of the ingested P was retained; the excess dietary P was, however, less in those experiments than in the work now described.

We are indebted to Mr H. Burton and Miss H. R. Chapman for the preparation of the milks and to Dr E. I. Garvie for freeze-drying them.

Table 2. *Weight gain and intake, excretion and retention of* Ca *and* P *from raw milk and from* U.H.T.*-treated milk before and after in-bottle sterilization*

(Mean values for groups of six litter-mate rats in an experiment lasting for 23 days.)

Type of milk	Weight gain, g	Diet eaten, g	Ca eaten, mg	Ca excreted		Ca retained		P eaten, mg	P excreted		P retained	
				Urine, mg	Faeces, mg	mg	%		Urine, mg	Faeces, mg	mg	%
Raw	96	255·28	323·8	2·9	2·5	318·5	98·4	1189·7	816·5	57·7	315·5	26·6
U H.T.	101	270·46	340·5	2·8	2·8	334·9	98·4	1245·3	882·4	61·4	301·5	24·3
U H.T. + in-bottle sterilization	101	274·10	341·9	2·8	2·5	336·7	98·5	1290·0	893·7	58·2	338·1	26·2
Standard error of the mean (10 D.F.)	—	—	—	—	—	—	±0·09	—	—	—	—	±0·65

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Studies on the manufacture of sweet cream starter butter

I. Effect of cream-holding temperature

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SUMMARY. Batches of cream inoculated with 1 % of a culture of *Streptococcus diacetilactis* or of one or other of two mixed starters were held at different temperatures over the range 37–57 °F before churning. Diacetyl and acetoin were estimated in the creams at churning and in the butters fresh and after storage.

The diacetyl production of all three starters in the cream increased with increase in temperature, although at the lower temperatures there was no indication of any rise in titratable acidity. At 52 and 57 °F there was some rise in acidity during the holding period.

The butters were of good keeping quality. There was a small but definite reduction in the diacetyl content of the butters during storage, but the extent of the change was not related to the holding temperature of the creams.

It was concluded that if sweet cream starter butter is to be made with uniform intensity of flavour the proportion of starter added must be adjusted to counterbalance changes in the cream-holding temperature.

The manufacture of butter from fully ripened cream, a common practice in Denmark and other European countries and at many factories in the United States of America, is not applicable to the manufacture of butter for export from New Zealand and Australia, for several reasons. (1) Butter made from ripened cream, especially salted butter, has poor keeping quality, owing to early development of oily and fishy flavours during storage at 14 °F. (2) The main market for the butter exported from these countries is for sweet cream butter, and it is necessary to maintain the supply to this market. (3) Manufacture of ripened cream butter would require the handling of cream of much lower fat content, which would greatly increase the manufacturing costs, especially in the large factories. (4) There would be difficulties in the production of a satisfactory buttermilk powder from the acid buttermilk. (5) The preference of the local market is for a sweet cream butter. Production of ripened cream butter for export along with production of sweet cream butter for local butter supply would introduce complications in butter factory operation.

The manufacture of a sweet cream starter butter differs from the manufacture of a ripened cream butter in that the temperature of holding of the cream is so low that there is no appreciable rise in acidity during the holding period. This has the advantage that the cream-cooling conditions used in the manufacture of sweet cream non-starter

butter are applicable, and facilities for raising the temperature of the cream to 65 °F (for incubation of the starter overnight) and for the subsequent cooling to churning temperature are not required. Since there is little increase in acidity in the cold cream during the holding period the resultant butter has a pH not markedly different from that of sweet cream butter. It maintains a fairly constant pH during storage and therefore is not subject to the 'oiliness' and 'fishiness' defects so commonly encountered in ripened cream butter after low-temperature storage.

For the ripening of the cream in the making of acid-cream butter it is usual to employ a mixed starter culture containing strains of *Streptococcus lactis*, *Str. cremoris* and *Leuconostoc*. At the temperature of incubation of the starter in the cream, usually 65 °F, the main effect of the streptococci in the mixed starter is to produce acid. When the pH of the cream has been lowered to below 5·0 the leuconostocs then produce diacetyl, acetoin and a number of other substances from the lactose and citric acid present. The main flavouring substance formed by the starter in the ripened cream, and so introduced into the butter, is diacetyl, which may be present in the butter in concentrations of up to 2·0 ppm. When the starter is added to cold cream, as in the making of sweet cream starter butter, there is, despite the absence of any marked rise in acidity, a significant production of diacetyl. Under these low-temperature conditions a pure culture of *Str. lactis* or *Str. cremoris* may produce more diacetyl than mixed cultures containing leuconostocs (Fabricius & Hammer, 1937; Wiley, Cox & Whitehead, 1939). The mechanism of this production of diacetyl and acetoin in the cold by different strains of starter bacteria has not been closely studied.

Use of diacetyl or of preparations containing diacetyl is common and legally acceptable for flavour improvement of margarine, and in some countries it is also permitted for flavour intensification in butter. In many countries, however, addition of synthetic diacetyl to butter is illegal, although increase of diacetyl content in the butter through use of starter cultures or genuine starter distillates is permitted. The purpose of the work described in the present series of papers was to explore the possibility of using starters for the improvement of the flavour of sweet cream butter, and to compare the results with those obtained by using diacetyl and commercial so-called 'dairy aromas'.

Temperature of incubation

The cooling conditions applied to cream in the making of sweet cream non-starter butter are chosen to provide control of churning time to within the range 30–45 min with a normal load of cream in the churn, and control of the physical properties of the butter. At some factories the cream after pasteurization is cooled directly to churning temperature and is delivered to the holding-vat at these temperatures. The requirement for refrigerated cooling in the holding-vat is then only that needed to maintain the temperature against the latent heat released during crystallization of the butterfat, which occurs mainly in the first 2 h (McDowall, 1953), and against absorption of heat from the atmosphere. At other factories it is general practice to take the cream off the cooler at somewhat above churning temperature and to complete the cooling in the refrigerated holding-vat.

At every factory, too, some change in the system of cooling of the cream is necessary with progress of the dairying season. In New Zealand when the cows are in the early

stages of lactation and the pastures are in the first flush of spring growth the butterfat is soft (Cox & McDowall, 1948), and a rapid cooling to a low temperature is necessary to give a sufficiently firm body to the butter. Because at this time the cream churns readily, cooling to this low temperature is necessary also to control the churning time and the extent of fat loss in the buttermilk. As the butterfat hardens with progress of the season it becomes necessary to take the cream off the cooler at a somewhat higher temperature and to effect the remainder of the cooling in the refrigerated holding-vat, in order to avoid production of butter with an over-firm brittle body and with colour defects such as 'packer streak'. In the autumn months this final slow cooling is required, also, to prevent the production of butter with a 'sticky' body.

Apart from the changes in cream-holding temperatures required for control of physical properties of butter, a steady rise in churning temperature is needed during the latter part of the season to keep the churning time within the normal range. The extent of this rise in temperature of churning varies from factory to factory.

In the making of sweet cream starter butter the variations in conditions of tempering of the cream are at the same time variations in the conditions of incubation of the starter in the cream, since the starter is usually added to the cream either in the cooler balance-tank or in the holding-vat as it is being filled. At factories where the cream is cooled on the refrigerated cooler directly to churning temperature the temperature conditions are less favourable for bacterial action than at factories where part of the cooling is applied in the holding-vat—in these latter factories the starter during the first few hours is incubated at a higher (but steadily falling) temperature.

The temperature of incubation used by Wiley *et al.* (1939) in their studies on diacetyl production by starters in the cold was 7 °C (45 °F). At this temperature they found no appreciable rise in acidity in the cream during the holding period. Information is required, however, on the effect of variation in incubation temperature within the range of the common cooling, holding and churning conditions in butter factories, as outlined above, on the diacetyl and lactic acid production of starter and on the quality of the butter.

EXPERIMENTAL

Sweet cream collected from farms daily was used. Before pasteurization it was treated with a small quantity of sodium bicarbonate neutralizer. The creams to be held at the higher temperatures were neutralized to a somewhat lower acidity (see Table 1), to allow for a possibly increased development of acidity in the cream at these temperatures. The creams, after Vacreator treatment, were taken off the refrigerated cooler at temperatures chosen to give the desired range of final temperatures. The holding-vats used were water-jacketed, the water being cooled by direct expansion ammonia coils in the jacket. The vats were not provided with mechanical agitators. During the first few hours the operator was present to control the cooling, but during the night it was necessary to rely on the residual ice in the water jacket to off-set the absorption of heat from the atmosphere, and this resulted in some fluctuation in the holding temperature of the cream overnight. In the absence of mechanical agitation there may also have been during this period some variations in temperatures within the vat of cream.

Since only one churn was available for the three comparative churnings each day it was not possible to churn the creams simultaneously. To obviate variation in time of contact of the starter with the cream the times of addition of starter to the vats were separated by intervals corresponding with the expected intervals between times of churning on the following day.

Table 1. *Cooling and cream acidity records for the separate vats of cream inoculated with 1 % of different starter cultures and held at different temperatures*

Trial and churning no.	Starter acidity, % lactic acid	Desired holding temp., °F	Temperature at beginning and during holding, °F. Times in hours given in brackets	Churning temp., °F	Acidity of cream, % lactic acid: After adding starter	At churning	Rise during holding
I–1	0·78	37	37, 35 (20)	42	0·07	0·07	0
2		42	42, 39 (20)	42	0·07	0·07	0
3		47	45, 45 (20), 42 (20½), 45 (21)	43	0·07	0·07	0
II–1	0·55	42	45, 39 (7), 41 (10), 42 (20)	49	0·065	0·07	0·005
2		47	48, 45 (7), 47 (10), 47 (20)	49	0·065	0·07	0·005
3		52	53·5, 52 (6), 52 (9), 52 (19)	49·5	0·055	0·06	0·005
III–1	0·80	47	41·5, 47 (4), 45 (14), 47 (21)	48·5	0·09	0·095	0·005
2		52	51·5, 53 (4), 54 (20), 47 (22)	48	0·08	0·09	0·01
3		57	56, 58 (3), 56 (20), 45 (23)	48	0·05	0·105	0·055
IV–1	0·81	37	37·5, 36 (5), 35 (13), 35 (19)	44	0·07	0·07	0
2		42	42·5, 40 (3), 41 (9), 41 (20)	42·5	0·07	0·07	0
3		47	47·5, 46 (5), 45 (15), 41 (20)	42·5	0·07	0·07	0
V–1	0·88	42	41, 40 (5), 43 (15), 45 (18)	51	0·085	0·085	0
2		47	46·5, 46 (2), 45 (8), 47 (17)	51	0·085	0·085	0
3		52	53, 53 (5), 52 (8), 52 (17)	50	0·075	0·095	0·02
VI–1	0·86	47	47·5, 46 (3), 46 (6), 47 (17)	48	0·09	0·10	0·01
2		52	51·5, 53 (7), 53 (12), 47 (19)	48	0·09	0·10	0·01
3		57	55·5, 56 (7), 55 (16), 45 (20)	48	0·04	0·09	0·05
VII–1	0·79	37	37, 37 (4), 36 (12), 37 (15)	50·5	0·08	0·08	0
2		42	43·5, 42 (2), 45 (15), 44 (17)	50	0·08	0·08	0
3		47	47, 48 (2), 46 (15), 48 (19)	50	0·08	0·08	0

The temperatures of holding varied from 37 to 57 °F in steps of 5 °F. This range was covered in a series of three temperature comparisons. The creams on any one day were brought to approximately the same temperature for churning.

The starters were prepared in skim-milk which had been pasteurized by heating at 200 °F for a few minutes, and they were 1 day old when added to the cream. For all the comparisons they were added in the proportion of 1 % of the volume of the cream. There were seven 3-churning trials, with starters as follows:

Trials I–III. *Str. diacetilactis.*

Trials IV–VI. A commercial mixed culture in common use in New Zealand and known as *Camb.*

Trial VII. A commercial culture F.D.

Diacetyl and acetoin were estimated in the starters at the time of adding them to the cream, in the creams at time of churning, in the fresh butters, and in the butters after 4 and 8 months' storage. The method used was the modification by Cox & Wiley (1939) of the method of Pien, Baisse & Martin (1937). The tests used for examination

of the butterfats from the stored butters were the ferric thiocyanate peroxide test as described by Loftus Hills & Thiel (1946), the Schibsted fat aldehyde test as modified by Mummery (1947), and the normal fat acidity test.

RESULTS

Effect on acidity of the cream at churning. The results in Table 1 show that at 37 and 42 °F there was no measurable rise in acidity during contact of the starter with the cream for 18–20 h. At 47 °F there was no rise in four of the seven trials, and in the remaining three (nos. II, III and VI) the rise recorded was 0·005–0·01 % lactic acid, which is almost within the range of experimental error of the acidity estimation. At 52 °F (trials II, III, V and VI) the rise was within the range 0·005–0·02 % lactic acid, whereas at 57° F there was a rise of 0·05–0·055 % in acidity during the holding period.

Table 2. *Diacetyl and acetoin contents of creams at churning and of fresh butters from creams treated with* 1 % *of starter and held at different temperatures*

Trial and churning no.	Diacetyl (D) and acetoin (A) in starter, ppm.	Holding temp., °F	Diacetyl, ppm.		Acetoin, ppm.		Diacetyl/ acetoin ratio in cream
			Cream	Butter	Cream	Butter	
I–1	D, 0·40	37	0·34	0·07	4·50	0·76	0·076
2	A, 270	42	0·38	0·07	5·90	0·82	0·064
3	—	47	0·56	0·09	7·20	1·24	0·078
II–1	D, 0·56	42	0·20	0·04	3·20	0·58	0·063
2	A, 138	47	0·20	0·045	4·20	0·70	0·048
3	—	52	0·46	0·09	9·00	1·52	0·051
III–1	D, 0·30	47	0·65	0·12	9·20	1·75	0·071
2	A, 203	52	0·91	0·14	17·0	2·40	0·054
3	—	57	1·05	0·24	28·5	5·45	0·037
IV–1	D, 2·28	37	0·12	0·04	3·30	0·66	0·036
2	A, 260	42	0·15	0·045	4·90	0·70	0·031
3	—	47	0·23	0·05	5·70	0·96	0·040
V–1	D, 0·24	42	0·17	0·04	5·20	0·90	0·033
2	A, 283·0	47	0·24	0·055	6·80	1·20	0·035
3	—	52	0·59	0·16	16·30	2·64	0·036
VI–1	D, 0·38	47	0·24	0·05	6·0	1·10	0·040
2	A, 268	52	0·48	0·105	12·50	2·05	0·038
3	—	57	0·32	0·075	10·0	1·65	0·032
VII–1	D, 0·58	37	0·13	0·035	2·60	0·58	0·050
2	A, 158	42	0·17	0·04	3·20	0·72	0·053
3	—	47	0·24	0·06	4·30	0·92	0·056

Effect on diacetyl and acetoin production. The results are shown in Table 2. The extent of formation of these substances in the creams during the holding period with one type of starter at any one temperature varied somewhat from day to day. In trial I, for example, *Str. diacetilactis* produced 180 % more diacetyl and 100 % more acetoin at 47 °F than in trial II. The temperature records for the creams show that this was not due to the cream in trial I being held at a higher temperature. It may have been due to variation in the vitality of the starter at time of addition, or to some variation in the conditions in the cream, e.g. extent of aeration (Wiley *et al.* 1939). Despite these irregularities there are in Table 2 clear indications that increase in

temperature of incubation gave increase in diacetyl production even when there were no measurable differences in lactic acid production (Table 1). At 37 °F, at which there would be negligible growth of the culture, the diacetyl production with the 1% addition of *Str. diacetilactis* (trial I, Table 2) was sufficient to give a diacetyl content of 0·34 ppm. in the cream and 0·07 ppm. in the butter.

There was a wide variation in the content of diacetyl in the starters at the time of their addition to the cream, probably due to destruction of diacetyl after its formation. With one or two exceptions production of diacetyl at all temperatures was greater with the strain of *Str. diacetilactis* used (trials I–III, Table 2) than with the mixed cultures (trials IV–VII). Except for trial II this applied also for acetoin. There was no consistent change in the diacetyl/acetoin ratio with change in temperature of incubation, but for any given temperature this ratio was higher for *Str. diacetilactis* than for the mixed cultures.

Table 3. *Diacetyl and acetoin contents of butters, fresh and after* 4 *and* 8 *months' storage, from creams inoculated with* 1% *of starter and held at different temperatures*

Trial and churning no.	Holding temp., °F	Diacetyl content of butter, ppm.			Acetoin content of butter, ppm.			pH of the fresh butter
		Fresh	4 mths	8 mths	Fresh	4 mths	8 mths	
I–1	37	0·07	0·07	0·065	0·76	0·82	0·92	6·70
2	42	0·07	0·06	0·06	0·82	1·02	0·96	6·70
3	47	0·09	0·075	0·075	1·24	1·52	1·16	6·75
II–1	42	0·04	0·055	0·055	0·58	0·56	0·92	6·79
2	47	0·045	0·05	0·05	0·70	0·74	0·96	6·70
3	52	0·09	0·09	0·095	1·52	1·46	1·16	6·75
III–1	47	0·12	0·10	0·12	1·75	1·80	1·80	6·41
2	52	0·14	0·14	0·14	2·40	2·40	2·35	6·60
3	57	0·24	0·20	0·19	5·45	5·50	5·90	6·63
IV–1	37	0·04	0·035	0·04	0·66	0·66	0·78	6·72
2	42	0·045	0·035	0·04	0·70	0·74	0·92	6·77
3	47	0·05	0·045	0·045	0·96	0·96	1·04	6·63
V–1	47	0·04	0·035	0·035	0·90	0·92	0·94	6·72
2	47	0·055	0·045	0·05	1·20	1·48	1·32	6·75
3	52	0·16	0·11	0·11	2·64	2·72	2·96	6·61
VI–1	67	0·05	0·055	0·04	1·10	1·05	1·12	6·69
2	52	0·105	0·07	0·07	2·05	1·50	1·92	6·90
3	57	0·075	0·085	0·08	1·65	1·95	2·04	7·23
VII–1	37	0·035	0·02	0·015	0·58	0·62	0·62	6·69
2	42	0·04	0·02	0·025	0·72	0·64	0·86	6·62
3	47	0·06	0·035	0·04	0·92	0·86	1·14	6·58

Diacetyl and acetoin content of the butters. From Table 2 it is evident that there was a general correspondence between the concentrations of diacetyl in the creams and the concentrations in the fresh butters. (For physical reasons, which will be discussed in a subsequent paper, an exact relationship is not to be expected.)

The results in Table 3 show that in most of the butters in which a fall in the diacetyl content occurred during storage the fall was only slight, and there was no significant or consistent change in the acetoin content. There was no indication of any relation-

ship between temperature of incubation of the starter in the cream during the holding period and change in diacetyl or acetoin contents of the butters during storage.

Grading of the butters and keeping quality tests on butterfats from the butters. In the grading of sweet cream starter butter the personal predilections of the grader accustomed to the grading of sweet cream non-starter butter can have an important influence. The full flavour obtained may not be attractive to the grader whereas it may be attractive to the consumer. The actual grades allotted to the experimental butters are thus of less importance than the changes in grade during storage of the butter. From the results in Table 4 there was no evidence of a relationship between the temperature of incubation of the starter in the cream and the grades of the butters

Table 4. *Grading results for butters, fresh and after* 4 *and* 8 *months' storage, made from creams inoculated with* 1 % *of starter and held at different temperatures*

Trial and churning no.	Starter used	Holding temp., °F	Grade and comments: Fresh butter		After storage for 4 months		After storage for 8 months	
I–1	*Str. diacetilactis*	37	92½	—	92½	—	92½	—
2		42	92½	—	92½	—	92½	—
3		47	92½	—	92	—	92	—
II–1	*Str. diacetilactis*	42	93	Slightly sour	92	—	90½	Slightly oily
2		47	93	—	93	—	90½	
3		52	93	Slightly fuller flavour	92	—	91	—
III–1	*Str. diacetilactis*	47	93	Preference for III–1	91½	Slightly sour	92	—
2		52	93		91½		92	—
3		57	93		91½		92	—
IV–1	*Camb*	37	93	—	93	—	92½	Storage flavour
2		42	93	—	93	—	92½	
3		47	93	—	92	Fuller flavour but less attractive	92	More storage flavour
V–1	*Camb*	42	93	—	93	—	93	Good flavour
2		47	93	—	92½	Fuller flavour	93	
3		52	93	Fuller flavour	92	Fuller but slightly oily	92	—
VI–1	*Camb*	47	93½	—	93	Preference for VI–1	92½	—
2		52	93½	Fuller flavour than 1	93	Light flavour	93	—
3		57	93	Fuller flavour than 2, weak body	92	Full flavour, soft body	92	—
VII–1	F.D.	37	93	—	92½	—	92	—
2		42	93	—	93	—	93	—
3		47	93	—	92½	—	92½	—

either fresh or after storage. The marked fall in grade in trial II for the butters after 8 months' storage, due to development of oily flavour, was probably accidental. The low grading for butter II-1 was supported by the high fat aldehyde and peroxide values for the butterfat (Table 5), but these values for butter II-2 were normal. Apart

from this sample the results in Table 5 are typical for butters of excellent keeping quality. The effect of high temperature of holding is apparent for butter VI-3, which was reported upon as having a 'weak' body.

Table 5. *Oxidation and acidity tests on butterfats from butters, after* 4 *and* 8 *months' storage at* 14 °F, *from creams treated with* 1 % *of starter and held at different temperatures*

Trial and churning no.	Holding temp., °F	Fat aldehyde value		Peroxide value		Fat acidity value, % oleic acid	
		4 mths	8 mths	4 mths	8 mths	4 mths	8 mths
I–1	37	0·28	0·20	0·20	0·24	0·11	0·10
2	42	0·34	0·20	0·24	0·24	0·11	0·11
3	47	0·31	0·20	0·20	0·20	0·11	0·10
II–1	42	0·45	0·59	0·36	0·48	0·12	0·12
2	47	0·28	0·24	0·20	0·20	0·12	0·12
3	52	0·31	0·26	0·20	0·24	0·12	0·11
III–1	47	< 0·2	0·28	0·04	0·20	0·15	0·15
2	52	< 0·2	0·24	0·04	0·20	0·15	0·14
3	57	< 0·2	< 0·2	0·04	0·14	0·16	0·15
IV–1	37	0·22	0·20	0·24	0·20	0·13	0·11
2	42	0·24	0·31	0·28	0·24	0·13	0·12
3	47	0·28	< 0·2	0·24	0·20	0·13	0·12
V–1	42	< 0·2	< 0·2	0·14	0·08	0·19	0·19
2	47	< 0·2	< 0·2	0·16	0·16	0·20	0·19
3	52	< 0·2	< 0·2	0·10	0·16	0·20	0·19
VI–1	47	< 0·2	< 0·2	0·04	0·12	0·21	0·21
2	52	< 0·2	< 0·2	0·04	0·14	0·21	0·21
3	57	< 0·2	< 0·2	0·04	0·12	0·19	0·18
VII–1	37	< 0·2	< 0·2	0·12	0·08	0·11	0·11
2	42	< 0·2	< 0·2	0·08	0·08	0·11	0·12
3	47	< 0·2	< 0·2	0·08	0·08	0·11	0·12

CONCLUSIONS

(1) Diacetyl production by starter cultures at normal temperatures of holding of cream for churning was shown to increase with increase in holding temperature from 37 to 57 °F.

(2) This increase with rise in holding temperature occurred even when there was no indication, from acidity and pH measurements, of any formation of lactic acid in the cream. Significant rises in acidity of the cream occurred only at the higher holding temperatures, 52 and 57 °F.

(3) At all the cream-holding temperatures investigated the single strain starter *Str. diacetilactis* produced considerably more diacetyl than the two commercial mixed cultures used. There was not the same difference in acetoin production.

(4) Diacetyl/acetoin ratio in the cream at churning was not related to temperature of incubation but it was greater with *Str. diacetilactis* starter than with the mixed starters used.

(5) The diacetyl contents of the butters ranged from 0·04 to 0·24 ppm. for the *Str. diacetilactis* churnings and from 0·04 to 0·16 ppm. for the mixed culture churnings.

(6) There was no relation between the temperature of incubation of the starter in the cream and the keeping quality of the butter.

(7) The results show that in making sweet cream starter butter it is necessary to adjust the proportion of starter added to accord with the temperature of the holding and churning of the cream if a butter of uniform intensity of flavour is to be produced.

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Studies on the manufacture of sweet cream starter butter

II. Effect of variation in proportion of starter added to cream

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SUMMARY. Production of diacetyl and acetoin by starters in cold skim-milk and cream was shown to increase with increase in the proportion of starter culture added, with some limitations at the higher rates of starter addition.

With *Streptococcus diacetilactis* starter in skim-milk at 50 °F the relation between proportion of starter added and production of diacetyl was linear up to addition at the 4 % level, whereas at 43 °F it was approximately linear up to the 10 % level. At both 50 and 43 °F the relation between the proportion of starter added and the production of acetoin was linear up to the 10 % level.

With *Camb* starter in skim-milk at both 50 and 43 °F there were regular increases in production of diacetyl up to the 4 % level of addition, but only minor changes thereafter with increase in the proportion of starter added up to 10 %. At both temperatures the maximum production of acetoin was reached with the 7 % rate of addition.

Production of diacetyl and acetoin in skim-milk was greater at 50 °F than at 43 °F with both starters for all proportions up to 10 %, and it was greater for *Str. diacetilactis* than for the mixed cultures.

Except at the higher rates of addition of starter and at the higher temperature there were no concomitant increases in the acidity of the milk or lowering of the pH values. It appears that at low temperatures production of diacetyl by starters in sweet milk and cream proceeds independently of production of lactic acid.

Similar results were obtained in a series of experimental buttermaking trials and some small commercial-scale trials, in which varying proportions of starter were added to creams after pasteurizing and before holding overnight for churning. With the cream-holding temperatures used, mainly 40–50 °F, the pH values of the butters were not appreciably lowered by the starter additions to the cream. At all the rates of addition there were with *Str. diacetilactis* starter higher contents of diacetyl in the butter than with *Camb* starter. There was no indication of any relationship between the proportion of starter added and the keeping quality of the butter.

In the manufacture of sweet cream starter butter the temperature of holding the cream for churning has been shown to have an important influence on the production of diacetyl in the cream during the holding period and so on the diacetyl content of the butter (McDowall, Singleton & Le Heron, 1960). It was concluded that if butters of uniform intensity of flavour are to be produced throughout the season the

proportion of starter added must be varied in accord with the variations in holding and churning temperatures. The present paper covers a study of the effect of variation in the proportion of starter added on diacetyl production in the cream held at uniform temperature, and on the quality and keeping quality of the butter.

Possible effects of variation in proportion of starter added

In the making of ripened cream butter, i.e. with incubation of the starter in the cream at 65 °F, increase in the proportion of starter added decreases the time required for the acidity to reach the value at which the leuconostocs begin to produce diacetyl. It thus leads to an earlier and more rapid production of diacetyl. If, however, the time of ripening is not carefully adjusted the diacetyl content of the cream may be past its maximum by churning time, owing to the subsequent destruction of diacetyl by the starter organisms in the fully ripened cream. Given adequate ripening time, therefore, increase in the proportion of starter added does not increase the final content of diacetyl in the cream. In making sweet cream starter butter, on the other hand, the object is to produce a butter with a pH approximately the same as that of sweet fresh cream. There is thus no alteration of the metabolism of the starter in the cream due to high acidity conditions.

Variation in the proportion of starter added to cold cream could cause a variation in the diacetyl and acetoin contents of the cream after holding: (*a*) because of change in the amount of already formed diacetyl and acetoin added; (*b*) because of the effect of change in the proportion of starter added on the pH of the cream (if diacetyl production by starters in the cold is affected by pH of the cream); and (*c*) because of a change in the rate of production of diacetyl and acetoin in the cream resulting from increase in the inoculation.

(*a*) *Pre-formed diacetyl in starter added.* The relation between the proportion of starter added and the increase in diacetyl and acetoin contents of the mixture due to the addition is not linear, because of the increase in the diluting effect of the starter with increase in the proportion of starter added. For the lower rates of addition the difference due to this diluting effect is small and within the range of the possible experimental error of the diacetyl and acetoin estimations, but for the higher rates of addition it is significant.

(*b*) *Effect of starter added on initial acidity and* pH *of cream.* An increase in the proportion of starter added to cream will result in an increase in the initial acidity of the cream after addition, and a lowering of the pH. The effects of different rates of addition of a starter on cream acidity will vary with the acidity of the cream to which it is added; and allowance must be made here also for the diluting effect of the starter added. Addition of 10 % of starter of acidity 1·08 % lactic acid to a cream of 0·08 % acidity would increase the acidity of the cream by 0·0918, i.e. to 0·17 %. The effect on the pH will vary with the fat content of the cream, and apart from this it will vary with the buffering capacity of the cream, due both to variation in the buffering capacity of undiluted cream plasma and to variation in the extent of dilution of the cream with water during processing in the butter factory. For a normal undiluted 40 % cream addition of 10 % of starter would reduce the pH immediately to about 6·0.

It is well known that salted butter of low pH has poor keeping quality. In the present investigations on the effect of the quantity of starter added on the quality and

keeping quality of butter the attempt was made to produce butters of uniform pH at time of manufacture. Allowance was made (i) for the rise in acidity caused by the acid in the starter added, and (ii) for the expected rise in acidity due to greater growth of the starter when present in higher proportion in the cream during the holding period, by neutralizing the cream to a lower acidity before adding the starter. The net result was a somewhat higher initial pH in the creams to which the larger proportions of starter were added, and this could possibly have affected the rate of diacetyl formation in the cream.

(*c*) *Variation in rate of diacetyl and acetoin production in cream.* Changes such as those discussed in (*a*) and (*b*) are the direct arithmetical result of the presence of preformed diacetyl, acetoin and lactic acid in the starter added. The effect of different rates of addition of starter on the actual production of these substances in the cream during the holding period and on the quality of the butter is the subject of the present paper.

It was found by Wiley, Cox & Whitehead (1939) that the production of diacetyl by starters in steamed skim-milk at 45 °F ran parallel with the production in cream, and that for laboratory work it was more convenient to use skim-milk. For the preliminary investigation on the effect of the proportion of starter on the production of diacetyl in the cold, therefore, skim-milk was used as the experimental medium.

EXPERIMENTAL

Trials with steamed skim-milk. Steamed skim-milk, 100 ml in each Erlenmeyer flask, was held in a cold incubator overnight, to permit exact adjustment to the temperature of the incubator. The acidities of the skim-milk and of the starter to phenolphthalein were then estimated, and 0·5 N-caustic soda solution was added to the skim-milk in the Erlenmeyer flasks in the quantities required to neutralize the lactic acid (but not the natural acidity in the starter milk) in the starter to be added. To the control and to the samples to which the smaller quantities of starter were to be added the appropriate volumes of distilled water were added to maintain the degree of dilution constant for all the samples. The starter culture, adjusted to the incubation temperature, was added in the desired proportion and mixed in by gentle shaking. (Care was taken to avoid aeration, which was shown by Wiley *et al.* (1939) to affect diacetyl production in cold milk and cream.) When incubation began the samples thus had the same acidity and pH but contained different proportions of starter. A portion of each sample was removed immediately for pH and acidity determinations, and the remainder was left in the cold incubator for 20 h. Acidity, pH, diacetyl and acetoin were then determined.

The times of addition of starter culture were spread at hourly intervals over a 4 h period, and the times of analysis were spread over a similar period in the same sequence, in order to maintain the time of contact of the starter with the milk constant for all samples. There was no attempt to allow for a possible effect of the extra time of holding of the starter culture for the later inoculations on the activity of the starter in the milk.

Comparisons were made with cultures of *Str. diacetilactis* and of the mixed starter *Camb*, as used in the earlier investigation (McDowall *et al.* 1960). The starters were

added in the proportions of 1, 2, 4, 7 and 10 % of the volume of skim-milk used. Trials were made with each starter at 50 and 43 °F.

Buttermaking trials. Cream treatment, inoculation with starter, and cream-holding and cream-churning conditions were as already described (McDowall *et al.* 1960). Comparisons were made with cultures of *Str. diacetilactis* added to the cream in proportions of $\frac{1}{2}$, $2\frac{1}{2}$ and 5 %. The creams to be treated with the larger inocula of starter were neutralized to a lower acidity. to compensate for the higher increase in acidity directly due to the added starter.

In addition to these direct comparisons, results are available for addition of varying proportions of starter to creams on separate days, in investigations on other aspects of the manufacture of sweet cream starter butter. These include investigations on the comparison of *Str. diacetilactis* with the mixed starter *Camb* at different rates of addition, and investigations on the use of chlorinated water for the washing of granules in the making of sweet cream starter butter with different rates of addition of both *Str. diacetilactis* and *Camb* cultures (McDowall *et al.* 1959). For these sets of churnings the same principles were followed as for the direct comparisons, namely neutralization to a lower acidity for the higher rates of starter addition.

Commercial scale trial. Samples of butter were taken from full-scale commercial churnings in a commercial trial in which different proportions of a mixed starter culture were used.

Analytical methods. Diacetyl and acetoin were estimated, as before, by the Cox & Wiley (1939) modification of the method of Pien, Baisse & Martin (1937). The tests used for examination of the butterfats from the stored butters were the ferric thiocyanate peroxide test as described by Loftus Hills & Thiel (1946), the Schibsted fat aldehyde test as modified by Mummery (1947), and the normal fat acidity test.

RESULTS

Trials with steamed skim-milk

The acidity and pH readings before and after holding are shown in Table 1. At 50 °F at the lower rates of addition of both *Str. diacetilactis* and *Camb* starters there were small but definite rises in acidity, with corresponding falls in pH value, and with addition of 7–10 % of starter there were rises in acidity of 0·03–0·05 % lactic acid and falls in pH of 0·2–0·3. The changes were of the same order for both starters used. At 43° F the changes in acidity and pH were very much smaller and tended to be of practical significance only for the 7 and 10 % starter additions. They were somewhat greater for *Camb* than for *Str. diacetilactis* starter.

With the higher rates of addition of starter and holding at 50 °F there was sufficient acid production to reduce the pH to a value which in a salted butter would be almost the danger level for development of fishiness, despite the prior addition of neutralizer to compensate for the acid present in the starter itself at time of addition. When in commercial practice these large proportions of starter are added to cream for the making of salted butter, therefore, it is clearly necessary to neutralize the cream to a considerably lower pH than that of normal fresh cream, to allow for the effects of the acidity of the starter added and for increases in acidity during the holding period. The

extent of over-neutralization required will vary with variations in the starter acidity and with the temperature of holding of the cream (see also McDowall *et al.* 1960*a*).

The contents of diacetyl and acetoin in (i) the starter at the time of mixing with the first sample of milk each day and (ii) in the milks after they had been held at the specified temperature for 20 h, and (iii) the increases in diacetyl and acetoin contents (computed from (i) and (ii)) after allowing for the quantities present in the starter itself, are given in Table 2, and the relationship between the proportion of starter added and the quantity of diacetyl and acetoin formed is shown in Fig. 1.

Table 1. *Acidities and* pH *values of milk—starter mixtures before and after holding for* 20 *h at low temperatures*

Code letter and proportion of starter added, %	Starter used	Temp. of holding, °F	Acidity, % lactic acid			pH value		
			Before holding	After holding	Difference	Before holding	After holding	Difference
A–0	*Str. diacetilactis*	50	0·150	—	—	6·80	—	—
1			0·145	0·158	+0·013	6·79	6·75	−0·04
2			0·150	0·163	+0·013	6·80	6·74	−0·06
4			0·150	0·173	+0·023	6·80	6·67	−0·13
7			0·148	0·188	+0·040	6·81	6·60	−0·21
10			0·147	0·193	+0·046	6·83	6·55	−0·28
B–0	*Str. diacetilactis*	43	0·158	—	—	6·78	—	—
1			0·160	0·159	−0·001	6·81	6·79	−0·02
2			0·160	0·160	0·00	6·78	6·79	+0·01
4			0·155	0·159	+0·004	6·81	6·80	−0·01
7			0·153	0·161	+0·008	6·82	6·80	−0·02
10			0·153	0·163	+0·010	6·79	6·79	−0·0
C–0	*Camb*	50	0·160	—	—	6·73	—	—
1			0·160	0·163	+0·003	6·76	6·72	−0·04
2			0·155	0·173	+0·018	6·82	6·65	−0·17
4			0·158	0·175	+0·017	6·80	6·67	−0·13
7			0·152	0·183	+0·031	6·84	6·59	−0·25
10			0·150	0·200	+0·050	6·83	6·53	−0·30
D–0	*Camb*	43	0·160	—	—	6·75	—	—
1			0·159	0·159	—	6·76	6·75	−0·01
2			0·157	0·158	+0·002	6·75	6·76	+0·01
4			0·160	0·161	+0·001	6·75	6·76	+0·01
7			0·160	0·176	+0·016	6·77	6.70	−0·07
10			0·150	0·168	+0·018	6·80	6·72	−0·08

At both 50 and 43 °F there was with *Str. diacetilactis* over the whole range of proportions investigated an increase in diacetyl production with increase in proportion of starter added. At 50 °F, however, the rate of increase in diacetyl content was smaller at the higher rates of starter addition, as indicated by the flattening of the top curve in A, Fig. 1. This change of rate of increase with increase in proportion of *Str. diacetilactis* starter added was not evident for acetoin at 50 °F (B, Fig. 1) or for either diacetyl or acetoin at 43 °F (A, B, Fig. 1).

With the mixed starter *Camb* at both temperatures the production of diacetyl was lower than with the single strain starter *Str. diacetilactis*, a finding which was in agreement with the earlier results (McDowall *et al.* 1960). At 50 °F there was with this starter little increase in diacetyl and acetoin production with rates of addition of

starter above 4% (C, D, Fig. 1). At 43 °F the effect of increase in proportion of starter added was still evident with the 7% addition but not with the 10% addition.

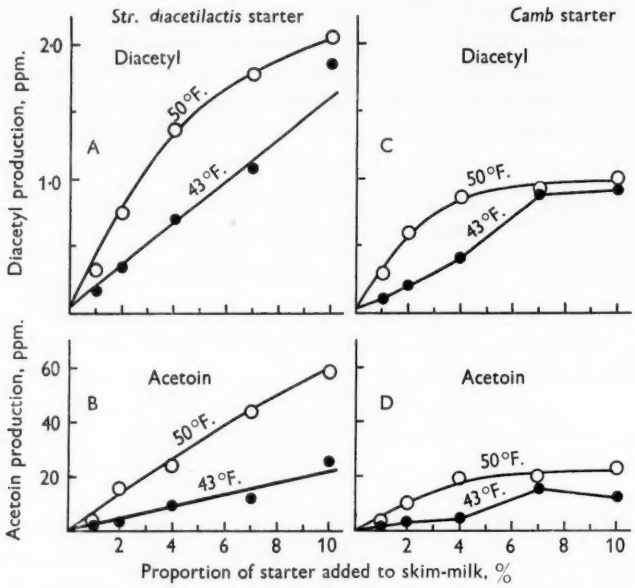

Fig. 1. The effect of the proportion of *Str. diacetilactis* and *Camb* starters on the diacetyl and acetoin production in skim-milk, after holding the milks at 50 °F and at 43 °F for 20 h.

Buttermaking trials

The following sets of results were available.

Trials I–III. Direct comparisons with *Str. diacetilactis* starter in different proportions.

Trials IV–VII. Churnings on different days with $\frac{1}{2}$, 2, 5 and 10% additions of *Str. diacetilactis* and of *Camb* starters.

Trials VIII–IX. Averages of three churnings on different days with 1% addition of *Str. diacetilactis* starter, and of three churnings with 5% addition.

Trials X–XI. Averages of three churnings on different days with 1% addition of *Camb* starter, and of three churnings with 5% addition.

Trial XII. Small-scale commercial trials of manufacture of butter with use of different proportions of a mixed starter.

In Trials I–III the same starter culture was added to cream in different proportions on the same day. The results therefore are a true comparison of the effects of proportion of starter added. In trials IV–XI, on the other hand, the investigation was being directed towards some other aspect of sweet cream starter butter manufacture, and the churnings with different percentage additions of starter were made on different days. Because of possible day-to-day variation in the diacetyl-producing activity of cultures of any one starter (McDowall *et al.* 1960) and of differences in the fat content and in the conditions of holding of the creams, these results for the dif-

ferent rates of addition of starter are not strictly comparable. They provide, however, a useful set of supplementary data.

Changes in acidity of cream. The results are given in Table 3. There were only minor increases in acidity of the cream during the holding period. This was so even with addition of 5 % of starter, in agreement with the previous findings for 1 % addition when the cream-holding and churning temperatures were as cited in Table 4 of the earlier paper by McDowall *et al.* (1960).

Table 2. *Diacetyl and acetoin contents of skim-milk after incubation with different proportions of starter and after correction for the diacetyl and acetoin in the starter added*

Code letter and proportion of starter added, %	Starter used and the diacetyl (D) and acetoin (A) contents, ppm.	Temp. of holding, °F	Diacetyl, ppm.		Acetoin, ppm.		Ratio of diacetyl produced to acetoin produced
			Total	Produced	Total	Produced	
A–0	*Str. diacetilactis*:	50	0·06	—	0·10	—	—
1	D, 1·90; A, 278		0·32	0·31	6·6	3·8	0·082
2			0·78	0·74	21·5	15·8	0·047
4			1·44	1·37	36·0	24·3	0·056
7			2·05	1·93	63·0	44·8	0·042
10			2·30	2·13	84·0	58·6	0·036
B–0	*Str. diacetilactis*:	43	0·03	—	0·14	—	—
1	D, 0·82; A, 257		0·16	0·15	3·5	0·9	0·167
2			0·36	0·34	8·5	3·3	0·103
4			0·72	0·69	19·0	9·2	0·075
7			1·12	1·07	28·0	11·3	0·095
10			1·85	1·78	48·0	25·0	0·071
C–0	*Camb*:	50	0·03	—	0·06	—	—
1	D, 0·50; A, 143		0·31	0·30	5·2	3·8	0·079
2			0·60	0·59	13·0	10·2	0·058
4			0·88	0·86	24·0	18·7	0·046
7			0·95	0·92	29·0	19·7	0·047
10			1·05	1·00	36·0	23·1	0·043
D–0	*Camb*:	43	0·03	—	0·10	—	—
1	D, 0·48; A, 153		0·11	0·10	2·2	0·7	0·143
2			0·21	0·20	4·8	1·8	0·111
4			0·40	0·38	9·4	3·7	0·102
7			0·40	0·37	25·0	15·2	0·057
10			0·96	0·91	25·5	11·7	0·078

Diacetyl and acetoin in cream and fresh butter. The results are given in Table 4. In churning I–3 addition of 2 % of *Str. diacetilactis* culture to cream held overnight when the temperature of the cream was 45 °F at the beginning, fell to 40 °F and then rose to 47 °F, yielded as high a final concentration of diacetyl in the cream as was present in the original ripe starter culture, and in churnings II–2 and III–2 addition of 2½ % of *Str. diacetilactis* culture to cream held overnight at approximately 50 °F gave a considerably higher final concentration of diacetyl than was present in the original starter. For the latter two churnings the rise in acidity of the cream was 0·01–0·015 % lactic acid (see Table 3). In churnings II–3 and III–3 addition of 5 % of *Str. diacetilactis* starter to the cold cream gave diacetyl contents of 0·53 and 0·38 ppm. respectively in the butter, which is representative of a butter with a quite strong diacetyl flavour. The results for churnings IV–XI were similar in character.

As in the earlier investigations (McDowall *et al.* 1960) and in the experiments with

skim-milk described above, the diacetyl production in the cold cream was considerably greater with *Str. diacetilactis* (IV–2 to VII–2, VIII, IX) than with the mixed starter *Camb* (IV–3 to VII–3, X, XI); and addition of *Camb* starter in proportions greater than 5 % did not result in any increase in the diacetyl content of the cream and butter.

Table 3. *Effect of different proportions of starter culture added to creams after pasteurization and cooling on acidity of cream at churning*

		Acidity, % lactic acid						
Churning no.	Proportion of starter added, %	Cream off cooler	Cream after addition of starter	Cream at churning	Increase during holding	Temperature, °F: Holding*	Temperature, °F: Churning	Fat in cream at churning, %
Str. diacetilactis								
I–1	0	0·075	—	0·075	—	44, 41, 45	47	38·5
2	½	0·075	—	0·075	—	44, 40, 45	47	38·5
3	2	0·075	—	0·075	—	45, 40, 46	47	38·5
II–1	½	0·06	0·065	0·07	0·005	52, 45, 50	51	41·5
2	2½	0·04	0·065	0·065	0·0	52, 45, 50	51	40
3	5	0·025	0·060	0·07	0·01	53, 45, 51	50	39
III–1	½	0·06	0·065	0·07	0·005	52, 46, 52	50	38
2	2½	0·04	0·065	0·07	0·005	51, 43, 50	50·5	38
3	5	0·025	0·06	0·08	0·02	51, 44, 51	50·5	37
IV–2	½	0·06	—	0·065	—	—	50	35·5
V–2	2	0·06	—	0·075	—	—	50·5	36
VI–2	5	0·035	—	0·08	—	—	50	38
VII–2	10	0·015	—	0·10	—	—	49·5	34·5
Camb								
IV–3	½	0·06	—	0·07	—	—	50	35·5
V–3	2	0·06	—	0·08	—	—	50	36
VI–3	5	0·035	—	0·10	—	—	49·5	38
VII–3	10	0·015	—	0·11	—	—	50	34
Str. diacetilactis								
VIII	1	—	0·078	0·087	0·009	45, 46, 50	50	35·5
IX	5	—	0·063	0·083	0·020	48, 43, 48	49	34·5
Camb								
X–2	1	—	0·083	0·083	0·00	48, 43, 44	46	37·5
XI–3	5	—	0·075	0·098	0·023	48, 41, 45	48	36·5

* The figures refer to the temperature at the beginning of holding, the minimum temperature during the night, and the temperature at the end of holding, respectively.

Acetoin content was increased by increases in the proportion of starter added, except for the 10 % addition of *Camb* starter, for which there was a fall in acetoin content (compare results for acetoin production in skim-milk, B, D, Fig. 1). The increases in the rates of addition of starter to the cream did not cause any consistent

change in the diacetyl/acetoin ratio in the butter (trials I–III, Table 4; for these trials results for the acetoin content of the creams at time of churning were not available).

Change in diacetyl and acetoin contents of the butters during storage. As in the previous investigation (McDowall *et al.* 1960) there was a falling off in the diacetyl content of the butter during storage at 14 °F for 4 and 8 months, but the extent of diacetyl loss was not related to the proportion of starter added to the cream (Table 5). As in the previous trials, also, there was a rise in the acetoin content of many of the butters during storage.

Table 4. *Effect of variation in the proportion of starter culture added to cream on the diacetyl and acetoin contents of the cream at churning and of the fresh butter*

Series no.	Starter used	Proportion of starter added, %	Diacetyl, ppm.			Acetoin, ppm.			Ratio of diacetyl to acetoin in butter	pH of butter
			Starter	Cream	Butter	Starter	Cream	Butter		
I-1	*Str. diacetilactis*	0	0·88	0·06	0·035	—	—	0·39	0·090	6·66
2		½	0·88	0·20	0·07	—	—	0·85	0·082	6·63
3		2	0·88	0·84	0·20	—	—	2·46	0·081	6·46
II-1	*Str. diacetilactis*	0	0·62	0·38	0·09	—	—	0·76	0·118	6·54
2		2½	0·62	1·16	0·23	—	—	2·92	0·079	6·72
3		5	0·62	2·15	0·53	—	—	5·45	0·097	6·58
III-1	*Str. diacetilactis*	0	0·76	0·38	0·05	—	—	0·54	0·091	6·92
2		2½	0·76	1·44	0·13	—	—	2·45	0·053	6·96
3		5	0·76	1·95	0·38	—	—	5·40	0·070	6·83
IV-2	*Str. diacetilactis*	½	1·38	0·24	0·05	146	2·8	0·52	0·096	6·78
V-2	*Str. diacetilactis*	2	0·56	0·90	0·16	172	11·2	2·00	0·080	6·82
VI-2	*Str. diacetilactis*	5	0·46	1·95	0·46	232	34·4	7·2	0·064	6·63
VII-2	*Str. diacetilactis*	10	0·44	2·60	0·60	255	71·0	9·8	0·061	6·45
IV-3	*Camb*	½	0·92	0·15	0·03	115	1·9	0·44	0·068	6·80
V-3	*Camb*	2	0·76	0·58	0·15	147	7·3	1·65	0·091	6·71
VI-3	*Camb*	5	0·94	1·40	0·31	161	26·0	7·6	0·041	6·62
VII-3	*Camb*	10	0·86	1·10	0·28	186	27·0	4·4	0·067	6·64
VIII	*Str. diacetilactis*	1	—	—	0·098	—	—	—	—	—
IX		5	—	—	0·35	—	—	—	—	—
X	*Camb*	1	—	—	0·049	—	—	—	—	—
XI	*Camb*	5	—	—	0·135	—	—	—	—	—

Grading of the butters. The results given in Table 6 show that increase in the proportion of starter added had no marked effect on the grade scores for the fresh butter. Where there were differences they could be attributed largely to the personal preference of the grader in respect to the fullness of the butter flavour.

For the butters in trials I and IV–XI there was no falling-off in flavour of the butters during storage which could be attributed to an increase in the proportion of starter added. An explanation of the lowering of grade for the butters in trials II and III after storage is not apparent. The pH values for butters in trial II were relatively low, but not lower than those in trial I, and those for trial III were well above the 6·50 value

usually accepted as the minimum if fishiness defects are to be avoided (McDowall, 1953).

The peroxide values, the Schibsted fat aldehyde values and the fat acidity values showed no evidence of any increase in deterioration of the butter due to inoculation of creams with large proportions of starter—the results obtained were of the same order as those given in Table 5 of the previous paper in this series (McDowall *et al.* 1960). From these butterfat quality tests there was no indication of any deterioration in the quality of the stored butters from trials II and III, for which there was a marked fall in grade. (As stated in the previous paper, onset of oily flavour is usually accompanied by a rise in both peroxide and fat aldehyde values.)

Table 5. *Effect of inoculation of creams with different proportions of starter cultures on the diacetyl and acetoin contents of the fresh butters and after storage for* 8 *and* 4 *months*

Series no.	Starter used	Proportion of starter added, %	Diacetyl content of butter, ppm.			Acetoin content of butter, ppm.		
			Fresh	4 months	8 months	Fresh	4 months	8 months
I–1	*Str. diacetilactis*	0	0·035	0·03	0·015	0·39	0·36	0·32
2		½	0·07	0·06	0·03	0·85	0·74	1·10
3		2	0·20	0·15	0·11	2·46	2·18	2·50
II–1	*Str. diacetilactis*	½	0·09	0·045	0·045	0·76	0·56	0·78
2		2½	0·23	0·19	0·17	2·92	3·60	3·90
3		5	0·53	0·38	0·38	5·45	8·60	9·00
III–1	*Str. diacetilactis*	½	0·05	0·04	0·05	0·54	0·96	0·78
2		2½	0·13	0·13	0·14	2·45	3·60	3·10
3		5	0·38	0·36	0·30	5·40	7·20	7·40
IV–2	*Str. diacetilactis*	½	0·05	0·04	0·04	0·52	0·60	0·64
V–2	*Str. diacetilactis*	2	0·16	0·14	0·13	2·80	3·00	3·00
VI–2	*Str. diacetilactis*	5	0·46	0·23	0·30	7·20	7·20	7·80
VII–2	*Str. diacetilactis*	10	0·60	0·52	0·47	9·80	11·40	11·60
IV–3	*Camb*	½	0·03	0·03	0·04	0·44	0·38	0·60
V–3	*Camb*	2	0·15	0·11	0·12	1·65	2·30	2·45
VI–3	*Camb*	5	0·31	0·23	0·22	7·60	5·60	5·60
VII–3	*Camb*	10	0·28	0·22	0·19	4·10	4·80	5·00
VIII	*Str. diacetilactis*	1	0·097	0·083	0·076	—	—	—
IX	*Str. diacetilactis*	5	0·34	0·31	0·28	—	—	—
X	*Camb*	1	0·049	0·049	0·050	—	—	—
XI	*Camb*	5	0·135	0·130	0·130	—	—	—

Small-scale commercial trials. The results for the diacetyl contents of the butters are given in Table 7. There was an increase in the diacetyl content of the butter with increase in the proportion of starter added up to 4·5–5·5 %, but no increase with rise to 6·5 %.

DISCUSSION

It appears from the acidity and pH results obtained in the trials with skim-milk (Table 1) that diacetyl and acetoin production by both *Str. diacetilactis* and the mixed starter *Camb* proceeded without simultaneous production of lactic acid. It is not

known whether the formation of diacetyl at low temperatures is caused by the action of enzymes already present in the starter or whether it is caused by a change in the metabolism of the starter organisms at the low temperature, leading to slow growth without production of lactic acid.

Table 6. *Grading results from butters when fresh and after 4 and 8 months' storage, made from creams inoculated with different proportions of starter before holding overnight at low temperatures*

Series no.	Starter used	Proportion of starter, %	Grade and comments: Fresh butter		After storage for 4 months		After storage for 8 months	
I–1	*Str. diacetilactis*	0	93½	—	94	—	93	—
2		½	93½	—	93½	—	93½	—
3		2	93½	—	93	Flavour too full	93	Flavour too full
II–1	*Str. diacetilactis*	½	93	—	91	Unsound flavour	90½	Unsound flavour
2		2½	93	—	90		91½	
3		5	92½	—	90½		91	
III–1	*Str. diacetilactis*	½	93½	—	93	—	92½	Mild bright flavour
2		2½	93	Flavour too full	92½	Flavour too full	92	Flavour too full
3		5	92½		91		92	
IV–2	*Str. diacetilactis*	½	93½	—	92	—	92	—
V–2	*Str. diacetilactis*	2	93	Bright full flavour	93	—	93	—
VI–2	*Str. diacetilactis*	5	93½		93½	No storage flavour	93½	No storage flavour
VII–2	*Str. diacetilactis*	10	93½		93½		93½	
IV–3	*Camb*	½	93½	—	92½	—	92½	—
V–3	*Camb*	2	93	Bright full flavour	93¼	—	93¼	—
VI–3	*Camb*	5	93½		93½	No storage flavour	93½	No storage flavour
VII–3	*Camb*	10	93½		93		93½	
VIII	*Str. diacetilactis*	1	93·67		93·17		92·83	
IX	*Str. diacetilactis*	5	93·17	Full flavour	93·00	Full flavour	92·72	Full flavour
X	*Camb*	1	93·50		92·83		92·28	
XI	*Camb*	5	93·00	Full flavour	93·00	Full flavour	92·25	Full flavour

Table 7. *Diacetyl contents of commercial factory butters made from creams inoculated with different proportions of a mixed starter culture*

Proportion of starter added, %	0·5	1·2	2·0	4·0	4·5	5·0	5·5	5·5	6·5
Diacetyl content of butter, ppm.	0·08	0·16	0·13	0·44	0·56	0·40	0·51	0·41	0·34

At the higher temperature and with the higher rates of addition of starter some production of acid did take place, and at the same time there was an increase in the quantity of diacetyl and acetoin produced. Here again it is not known to what extent the two changes are interdependent.

While in the buttermaking trials there was no differentiation between the amount of diacetyl present in the starter and so added to the cream at the time of inoculation and the amount of diacetyl already formed in the cream during the holding period, the results, so far as they go, indicate that in the making of sweet cream starter butter there is a limit to the increase in the content of diacetyl obtainable with increase in the proportion of starter added. The rate of starter addition at which the maximum diacetyl content in the butter is reached varies with the type of starter culture and with the temperature at which the cream is held.

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Restricted and *ad lib.* feeding of liquid skim-milk, with and without the addition of formalin, to fattening pigs

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Summary. Results are reported of an experiment with fattening pigs, made under commercial conditions, on the effect of adding formalin to liquid skim-milk, to delay souring, on the nutritive value of the milk given either in restricted or in unrestricted quantities.

There were four treatments: (i) Meal, fed to a scale based on live weight, plus sour skim-milk restricted to a maximum of 5¼ pints per pig per day. (ii) As (i) except that milk kept sweet by the addition of 0·15 % formalin shortly after separation, was fed. (iii) Daily allowance of 2 lb meal/pig fed throughout the fattening period plus an unrestricted supply of sour skim-milk. (iv) As (iii) except that milk kept sweet by the addition of 0·15 % formalin shortly after separation, was fed.

There were five pens of nine group-fed pigs on each treatment, involving a total of 180 pigs. The pigs were on experiment from about 9 weeks of age to bacon weight. Comprehensive carcass measurements were made on all the pigs, and bacon-tasting tests were made on samples of both green and smoked bacon from a number of the carcasses.

There were no significant differences between any of the four treatments in rate of growth or overall efficiency of food utilization.

Pigs given formalin-treated milk had a significantly thinner layer of back fat over the loin than those given sour milk. Although the difference was relatively small, it was apparent whether the milk was fed in restricted amounts or *ad lib.* and was reflected in the commercial grading results. There were no significant differences between treatments in dressing percentage, carcass length, shoulder back fat thickness, belly thickness or size of eye muscle.

No taint or off-flavour was detected in samples of green or smoked bacon from pigs fed milk to which 0·15 % formalin had been added.

The feeding of liquid skim-milk in unrestricted quantities is discussed in relation to the comparative prices of meal and skim-milk.

The importance of adding the formalin to the skim-milk as soon as possible after separation, particularly during warm weather, is stressed in order that souring might be delayed for at least 1 week.

Barber, Braude & Mitchell (1956) showed that the addition of 0·1 to 0·2 % of formalin* to sweet liquid skim-milk was effective in delaying souring for sufficient time to permit the feeding of consistently sweet milk under conditions where deliveries were made to the farm only once or twice weekly. Although it is well established that skim-milk may be fed to pigs either sweet or completely sour, this addition of formalin offered a means of allowing the milk to be piped direct to the troughs, normally impracticable with sour, clotted milk. It was also a promising means of overcoming the adverse effects associated with the feeding of only partially soured milk, a problem frequently encountered when 'sour' milk is fed in practice. Preliminary trials indicated that skim-milk with 0·1–0·2 % of formalin added could be satisfactorily fed to fattening pigs. The data obtained were limited, however, and the present experiment was made to obtain more information on the effect of such treatment on the nutritive value of the milk for fattening pigs.

It is recognized that to obtain the greatest economic value from skim-milk, it should be fed in amounts restricted to those needed to meet the protein requirement of the pigs, namely up to a maximum of about 5–6 pints per pig per day; for one of the comparisons between sour and formalin-treated milk investigated here, this restricted feeding was used.

In some countries, at certain seasons of the year, large supplies of liquid skim-milk become available at a price low enough to make it economic to feed very much larger quantities to fattening pigs, the milk then being used to supply substantial amounts of energy as well as protein. At the time when this experiment was planned, there were indications that large quantities of liquid skim-milk might become available in this country for certain periods at a price low enough to justify using it as an energy food, and it was felt that it would be of value to have information available on the effects of unrestricted feeding of both sour and formalin-treated skim-milk. It has been shown in New Zealand (Smith, 1956), where the skim-milk is often given as the sole source of food, that better results may be expected if the milk is not fed completely *ad lib.* but is restricted somewhat so that the daily ration in gallons is equivalent to about 3½–4 % of body weight. Thus, at 100 lb each pig receives about 3½–4 gal of skim-milk per day. Griffiths (1957) recommended that the milk should be given *ad lib.* if a daily ration of 1 lb of meal was also provided.

In the experiment reported here, the pigs received an unrestricted supply of liquid skim-milk, together with a daily allowance of 2 lb of meal/pig, in the anticipation that this would result in an automatic restriction to some degree of the milk intake and prevent the bad scouring troubles usually encountered when unrestricted milk is given as the sole source of food.

EXPERIMENTAL

Treatments and diets

There were four treatments.

(i) Meal, fed to a scale based on live weight, plus sour liquid skim-milk restricted to a maximum of 5¼ pints per pig per day.

(ii) As (i) except that milk kept sweet by the addition of 0·15 % formalin shortly after separation, was fed.

* 40% solution of formaldehyde gas in water.

(iii) A daily allowance of 2 lb of meal per pig fed throughout the fattening period plus an unrestricted supply of sour skim-milk.

(iv) As (iii) except that milk kept sweet by the addition of 0·15 % formalin shortly after separation, was fed. The same meal mixture was fed to the pigs on all four treatments, and the composition of this mixture is given in Table 1. The scale, based on live weight, for determining the amounts of meal and of milk that were given to the pigs on treatments (i) and (ii), is shown in Table 2.

Table 1. *Percentage composition of meal mixture given to all experimental pigs*

Barley meal	75
Ground wheat	23¾
Limestone flour	1
Salt	¼
Vitamealo 'Supercon' special supplement*	3½ lb/2000 lb

* Agricultural Food Products Ltd., containing in 3½ lb: 14 g oxytetracycline, 4 200 000 i.u. vitamin A and 1 050 000 i.u. vitamin D_3. Other B vitamins and trace minerals were included in this commercial product.

Table 2. *Meal and skim-milk scale for pigs on treatments* (i) *and* (ii)

Live weight, lb	Meal, lb	Skim-milk, pints	Live weight, lb	Meal, lb	Skim-milk, pints
40–42	1·8	3	103–105	3·6	5¼
43–45	1·9	3	106–108	3·7	5¼
46–48	2·0	3	109–111	3·8	5¼
49–51	2·0	3½	112–114	3·9	5¼
52–54	2·1	3½	115–117	4·0	5¼
55–57	2·2	3½	118–120	4·1	5¼
58–60	2·3	3½	121–123	4·2	5¼
61–63	2·4	4	124–126	4·3	5¼
64–66	2·5	4	127–129	4·4	5¼
67–69	2·6	4	130–132	4·5	5¼
70–72	2·7	4	133–135	4·6	5¼
73–75	2·7	4½	136–138	4·7	5¼
76–78	2·8	4½	139–141	4·8	5¼
79–81	2·9	4½	142–144	4·9	5¼
82–84	3·0	4½	145–147	5·0	5¼
85–87	3·0	5	148–150	5·1	5¼
88–90	3·1	5¼	151–153	5·2	5¼
91–93	3·2	5¼	154– slaughter	5·3	5¼
94–96	3·3	5¼			
97–99	3·4	5¼			
100–102	3·5	5¼			

Delivery, storage and distribution of skim-milk

The milk, which was not pasteurized after separation, was delivered from a local factory once weekly for about the first 2 months of the experiment and thereafter twice weekly. Four 1000 gal steel tanks were used for storing the milk at the farm, two being used alternately for the sour milk and two for the formalin-treated milk. Between each delivery the two tanks not in use were thoroughly scrubbed out with water, followed by scrubbing with a hypochlorite solution (as used for dairy utensils) and rinsing with water.

The first delivery of milk which was to be fed sour was inoculated with a mixed

cheese starter culture. Subsequently, a small quantity (about 1 % of the incoming delivery) of the soured milk from the previous delivery was put into the cleaned tank, to act as a starter, before the freshly delivered milk was pumped into the tank. Two days were allowed between delivery and feeding for souring to be completed, and it was found in practice that this interval was quite adequate for clotting to take place under the conditions prevailing.

The milk to be formalin-treated, which was always from the same bulk source as that to be fed sour, had 0·15 % of formalin (1½ gal/1000 gal milk) added to it immediately on delivery at the farm. About 3 weeks after the start of the experiment during a period of warm weather in July, the batches of formalin-treated milk began to go sour within 6 days of delivery. Special extra deliveries were arranged so that this soured milk should not have to be fed. At the same time it was arranged that the formalin should in future be added direct to the delivery tanker at the factory at the earliest possible moment after separation. It was found that under these conditions the milk remained sweet for at least 8 days, and this routine was continued for the remainder of the experiment. During the latter part of the experiment in October and November, it was found that the formalin-treated milk remained sweet for upwards of 12–14 days. As the total consumption of milk increased to 2000 gal/week about 7 weeks after the start of the trial, it became necessary, owing to the limitation of the tank storage capacity, to have the milk delivered twice weekly to the farm. At the same time, it had been observed that the consumption of formalin-treated milk by the pigs having an unrestricted supply available, tended to drop slightly when they were first given the newly delivered formalin-treated milk. Consequently, it was arranged that henceforth the formalin-treated milk should be allowed to stand in the tank for 2 days after delivery before being fed.

The distribution of the sweet and sour milks to the pigs was achieved by running off the required quantities from the storage tanks into 100 gal mobile open containers (one for the sweet milk and one for the sour). Pipes of 3 in. diameter were fitted to the bottoms of the storage tanks to prevent the clotted sour milk blocking the outlets. Before the sour milk was run off in this way, the milk in the tanks was thoroughly agitated to break up the clot and so prevent the liquid phase draining off leaving the solid phase behind in the tank. The mobile containers were then wheeled along the feeding passage and measured quantities of milk distributed in buckets to the pen troughs.

Pigs

All the pigs used, with the exception of one litter, were from cross-bred sows containing Large White, Landrace or Wessex Saddleback blood. The sires of all the litters used were either pure Large White or pure Landrace. The litters were reared on pasture with their dams and were weaned at 56 days (± 4 days), weaning being done on one day in each week. Both the sows and litters had unrestricted whey available to them during the suckling period. In addition, proprietary creep pellets containing an antibiotic were provided from 3 weeks of age.

After weighing at weaning the pigs were selected for experiment and brought into the Danish-type fattening house described below. Selection of the pigs was carried out as described by Braude, Clarke, Mitchell, Cray, Franke & Sedgwick (1957). There

were five pens of nine pigs on each of the four treatments, making a total of 180 pigs for the experiment. The four treatments were randomly allocated to the pens within each of the five replicates. The first replicate was selected on 12 June 1958 and the fifth replicate on 24 July 1958.

Housing

The western wing of the Danish fattening house, previously described by Braude *et al.* (1957), was used. A number of alterations, however, were made to the house before the present experiment began. The false ceiling of straw was replaced by a complete internal shell consisting of expanded polystyrene sheets fixed to a wooden framework. This extended across the pens and central feeding passage at a height of about 7 ft and continued down to the internal walls which separated the pens and dung passage. At each end of the house it was extended down to the internal pen walls. A forced ventilation system was also installed. Air was drawn in by two fans through two inlets, situated on the roof ridge at each end of the building, and blown into the house via openings over the central feeding passage. The door openings leading from each pen to the dung passages formed the air outlets. The ventilation fans were regulated thermostatically from a single thermostat which was situated centrally in the house and was set for the fan to cut in at a temperature of 65 °F. In addition, raised insulated concrete floors with a greater slope were installed in each of the pens, which replaced the wooden platforms and straw bedding used earlier (Braude *et al.* 1957).

Experimental feeding of pigs

For the first week after being put into the experimental pens, the pigs were fed the same creep pellets that they had received from 3 weeks of age, together with access to an unrestricted supply of whey which they had had since birth. Whey was stopped abruptly at the end of this first week, and skim-milk feeding commenced. For those pigs receiving restricted skim-milk the amounts given were according to the scale based on live weight shown in Table 2. The pigs on the *ad lib.* skim-milk treatments were restricted to a maximum of $\frac{1}{2}$ gal/pig for the first 3 days, to $\frac{3}{4}$ gal/pig for the next 3 days and then *ad lib.* for the remainder of the experiment. The change from creep pellets to the experimental meal shown in Table 1 was made gradually over the first 3 days of the second week.

The pigs on treatments (i) and (ii) (restricted skim-milk) were given their daily ration of meal and milk, mixed together in the trough, once daily in the afternoon. An unrestricted supply of water was continuously available to these pigs from automatic water bowls. The consumption of water was not measured.

The pigs on treatments (iii) and (iv) (unrestricted skim-milk) were given their daily allowance of 2 lb of meal at one feed in the afternoon. Skim-milk was added to the trough when the meal had been consumed, additional amounts being added as necessary to ensure that it was continuously available. As far as possible, the amounts of skim-milk given were adjusted so that only a small quantity remained when the daily meal ration was added to the trough each afternoon. Apart from the first week when the amount of skim-milk given was restricted, no supply of water was available to the pigs on these two treatments.

Records

All pigs were individually weighed once weekly in the morning. All food given to each pen of pigs was recorded, the meal being weighed and the skim-milk measured in pints. Each week, coinciding with the weighing day, refusals of meal or skim-milk were determined, so that records were available of the total weekly food consumption by each pen of pigs throughout the experiment.

The allowance of meal and skim-milk for the pigs on treatments (i) and (ii) was adjusted once weekly after weighing. The amounts of food for each pig in the pen, based on live weight and the scale shown in Table 2, were totalled to give the daily allowance of meal and milk for each pen of nine pigs for the following week.

When the total food consumption of each pen of pigs was determined at the end of the experiment, it was necessary to allow for the food eaten by the pigs that either died or had to be taken off the experiment (see later). As the pigs were group-fed, no exact figure of the food consumed by an individual animal could be obtained and hence an estimate was made using a simple proportion of the total for the pen involved. While this may have introduced some slight error, it was considered unimportant in view of the relatively small percentage of pigs involved. To obtain an over-all figure of food consumption per lb gain, the skim-milk consumed was converted to a 12% moisture basis, equivalent to the approximate moisture content of the meal portion of the ration. By using figures of 9·4% for the average dry-matter content of liquid skim-milk and 10·35 lb for the weight of 1 gal of liquid skim-milk, the 12% moisture equivalent for the skim-milk in pounds was obtained by multiplying the figure for liquid skim-milk in pints by 0·1382.

Carcass grading

When each pig reached a minimum live weight of 200 lb at the once weekly weighing, it was removed from the pen and sent to slaughter immediately without further feeding. All pigs went to the same local bacon factory and were killed soon after arrival. On the same day the carcasses were commercially graded on the basis of length and shoulder and loin fat thickness. In addition, the following measurements were taken, always by the same person, within 1–2 h of slaughter using the left-hand side of each carcass while hanging: back fat thickness over shoulder and over loin in the same positions as used for determining the commercial grading, and three measurements of belly thickness according to the method described by McMeekan (1940). The average of these three belly measurements was used to give an over-all assessment of streak thickness. On the day following slaughter, after approximately 24 h chilling, the left-hand side of each carcass was cut through at a point level with the last rib, and the length and depth (widest distance at right angles to the position of the length measurement) of the eye muscle were determined.

Bacon-tasting tests

Samples of both smoked and green bacon from a number of pigs on treatments (iii) and (iv) were tasted in pairs by a panel of consumers to determine whether any taint or off-flavour attributable to the formalin consumed by the pigs on treatment (iv) could be detected.

Health of the pigs

During the course of the experiment, sixteen of the total of 180 pigs, or just under 9%, either died or had to be taken off the experiment for the reasons given in Table 3. As in previous trials at East Farm, virus pneumonia symptoms were observed in the pigs and the same routine described previously (Braude *et al.* 1957) to control the secondary complications of this condition was applied. The numbers of pigs receiving at least one course of injections were 4, 3, 9 and 9 on treatments (i), (ii), (iii) and (iv) respectively. All pigs were sprayed with benzene hexachloride twice at weekly intervals shortly after they entered the fattening house as a precaution against sarcoptic mange.

Table 3. *Details of pigs that died or were taken off the experiment*

Treatment no.	No. of pigs involved	Cause of death or removal from experiment
1	2	Failed to grow normally
2	1	Died—acute peritonitis
	1	Lame
3	3	Failed to grow normally
	1	Died—cause unknown
	1	Broken leg
4	2	Died—erysipelas
	1	Failed to grow normally after erysipelas
	1	Died—cause unknown
	2	Lame
	1	Failed to grow normally

Approximately 3 months after the start of the trial an outbreak of swine erysipelas occurred in one pen of pigs on treatment (iv). The affected animals were promptly injected with penicillin and all the other pigs on the experiment were given an injection of erysipelas serum. Two to three weeks later further isolated cases occurred in other pens, two in one pen on treatment (iii), three in another pen on the same treatment and a further one on treatment (iv). These were all given penicillin injections and there were no further cases. With the exception of two pigs which died, and one which failed to do well subsequently, all affected pigs responded quickly to the penicillin treatment and within a week were all growing normally again. This trouble, no doubt, had the effect of reducing slightly the figure for the average rate of growth given in Table 4 of the pigs on treatments (iii) and (iv) receiving *ad lib.* skim-milk. However, in view of the rapid and marked response of all but three of the affected pigs to treatment, it was considered that inclusion of the data in the results of all the pigs involved in the erysipelas outbreak (except for the three as shown in Table 3) would not affect any conclusions to be drawn from the experiment.

RESULTS

Treatment means for live-weight gain, efficiency of food conversion and carcass measurements, together with their standard errors, are given in Table 4. The standard errors were calculated from randomized block analyses of variance on the pen means,

no adjustments being made for variation in either initial live weight or cold dead weight (Barber, Braude & Mitchell, 1957). The experiment was designed as a 2×2 factorial and the results of the appropriate analyses are given in Table 5 with the standard errors and statistical significance of the treatment effects and interactions. The commercial grading results are summarized in Table 6.

Table 4. *Treatment means, with their standard errors, for live-weight gain, efficiency of food conversion and carcass measurements of pigs on experiment from approximately 9 weeks of age to bacon weight*

	Treatment no.					
	(i) Restricted sour skim-milk	(ii) Restricted formalin-treated skim-milk	(iii) *Ad lib.* sour skim-milk	(iv) *Ad lib.* formalin-treated skim-milk	Standard error of means*	Significance of treatment mean square†
Number of pigs	43	43	40	38	—	—
Initial weight (lb)	50·8	50·4	51·4	51·0	—	—
Final weight (lb)	204·6	204·7	204·7	204·0	—	—
Days on experiment	112·8	112·5	105·1	110·9	—	—
Daily gain (lb)	1·39	1·39	1·48	1·39	0·025	N.S.
Total consumption of food/pig:						
Meal (lb)	431·7	430·8	209·6	222·1	—	—
Skim-milk (gal)	67·4	67·1	271·0	252·5	—	—
Meal + 88 % dry matter skim-milk (lb)	506·3	505·0	509·1	501·2	—	—
Consumption of food/lb gain:						
Meal (lb)	2·80	2·79	1·37	1·45	0·033	—
Skim-milk (gal)	0·44	0·44	1·77	1·65	0·023	—
Meal + 88 % dry matter skim-milk (lb)	3·29	3·27	3·33	3·28	0·049	N.S.
Dressing percentage	76·3	76·6	77·0	76·5	0·36	N.S.
Carcass length (mm)	791·4	795·0	793·4	797·2	2·6	N.S.
Shoulder back fat (mm)	50·3	50·6	51·8	50·6	0·69	N.S.
Loin back fat (mm)	31·4	30·7	32·5	30·1	0·64	N.S.
Belly thickness (mm) (average of 3 points)	41·2	41·0	41·2	40·9	0·29	N.S.
Eye muscle (mm)						
Breadth	81·4	81·2	80·4	81·2	0·48	N.S.
Depth	41·3	40·2	39·5	40·7	0·58	N.S.

* Based on 12 degrees of freedom. † Significance levels: N.S. $P > 0{\cdot}05$.

(*a*) *Live-weight gain and efficiency of food utilization*

Treatment differences in rate of growth and over-all efficiency of food utilization (meal + 88 % dry-matter skim-milk/lb gain) were small and were not statistically significant.

In accordance with the experimental treatments imposed, more meal and less milk were consumed by the pigs given restricted quantities of milk compared with those given access to unlimited quantities of milk. The figures in Table 4 also show that the

Table 5. *Treatment constants and their standard errors, and the statistical significance of the treatment effects and interactions*

	Sour *v.* formalin-treated skim-milk			Restricted *v. ad lib.* skim-milk				
	Sour (1)	Formalin-treated (2)	Significance of treatment mean square† (3)	Restricted (4)	*Ad lib.* (5)	Significance of treatment mean square† (6)	Standard error of means (7)	Significance of interaction† (8)
Daily gain (lb)	1·44	1·39	N.S.	1·39	1·44	N.S.	0·018	N.S.
Consumption of food/lb live-weight gain:								
Meal (lb)	2·09	2·12	N.S.	2·80	1·41	—	0·023	N.S.
Skim-milk (gal)	1·10	1·04	*	0·44	1·71	—	0·016	*
Meal + 88 % dry matter skim-milk (lb)	3·31	3·28	N.S.	3·28	3·30	N.S.	0·035	N.S.
Dressing percentage	76·7	76·6	N.S.	76·4	76·8	N.S.	0·25	N.S.
Carcass length (mm)	792·4	796·1	N.S.	793·2	795·3	N.S.	1·8	N.S.
Shoulder back fat (mm)	51·1	50·6	N.S.	50·5	51·2	N.S.	0·49	N.S.
Loin back fat (mm)	32·0	30·4	*	31·1	31·3	N.S.	0·45	N.S.
Belly thickness (mm) (average of 3 points)	41·2	41·0	N.S.	41·1	41·1	N.S.	0·21	N.S.
Eye muscle (mm)								
Breadth	80·9	81·2	N.S.	81·3	80·8	N.S.	0·34	N.S.
Depth	40·4	40·4	N.S.	40·7	40·1	N.S.	0·41	N.S.

Col. (1). Mean of treatments 1 and 3.
Col. (2). Mean of treatments 2 and 4.
Col. (3). Significance of difference between cols. 1 and 2.
Col. (4). Mean of treatments 1 and 2.
Col. (5). Mean of treatments 3 and 4.
Col. (6). Significance of difference between cols. 4 and 5.
Col. (7). Standard error of each of the means in cols. 1, 2, 4 and 5.
Col. (8). Significance of the interaction between amount and type of skim-milk.

† Significance levels: N.S. $P > 0{\cdot}05$; * $0{\cdot}05 > P > 0{\cdot}01$.

pigs given formalin-treated milk *ad lib.* consumed on average approximately 12 lb more meal and 18 gal less milk during the whole fattening period than those given sour milk *ad lib.*

(*b*) *Dressing percentage, carcass length, back fat and belly thickness, and eye muscle size*

Treatment differences were in the main all small and none was significant, with the exception that type of milk (sour or formalin-treated) had a small, although statistically significant, effect on loin fat thickness. Table 5 shows that pigs receiving formalin-treated milk had a significantly thinner layer of fat over the loin than those given sour milk. Table 4 shows that this difference was apparent both in the restricted and *ad lib.* comparisons. Differences between pigs fed restricted and those given *ad lib.* skim-milk were all small and were not significant (Table 5).

Table 6. *Commercial grading* results of pigs (percentages)*

		Final grade										Carcass length			
Treatment	No. of pigs	AA+	AA	A	Total A or above	B+	B	Total B+ and B	C	F	A on shoulder	A on loin	800 mm and above	775–799 mm	Below 775 mm
(i)	43	23·3	11·6	2·3	37·2	34·9	11·6	46·5	13·9	2·3	69·8	37·2	34·9	48·8	16·3
(ii)	43	23·3	20·9	2·3	46·5	32·6	4·6	37·2	13·9	2·3	65·1	48·8	41·9	48·8	9·3
(iii)	40†	23·1	5·1	2·6	30·8	38·5	5·1	43·6	25·6	0·0	64·1	35·9	46·2	33·3	20·5
(iv)	38	34·2	18·4	5·3	57·9	28·9	2·6	31·5	7·9	2·6	78·9	57·9	52·6	36·8	10·5
Sour milk		—	—	—	34·0	—	—	45·0	19·7	1·2	66·9	36·5	40·6	41·0	18·4
Formalin-treated milk		—	—	—	52·2	—	—	34·3	10·9	2·5	72·0	53·4	47·3	42·8	9·9
Restricted milk		—	—	—	41·8	—	—	41·9	13·9	2·3	67·5	43·0	38·4	48·8	12·8
Ad lib. milk		—	—	—	44·3	—	—	37·5	16·8	1·3	71·5	46·9	49·4	35·1	15·5

* As in force at the time when the pigs were slaughtered.
† Includes one pig which was not graded due to the presence of a tuberculous abscess in spine.

(*c*) *Commercial grading results*

The significantly thinner loin fat measurements of pigs fed formalin-treated milk compared with those given sour milk were reflected in rather better back-fat grading results, this being particularly marked in the percentage of A gradings over the loin. Differences in grading results between pigs fed milk restricted and milk *ad lib.* were small.

(*d*) *Bacon-tasting tests*

No taint or off-flavour was detected in the smoked or green bacon samples from carcasses of pigs fed the formalin-treated milk *ad lib.* Furthermore, bacon from all the experimental carcasses was distributed to retailers in the normal way and no complaints were received concerning its quality.

DISCUSSION

The results show that the addition of 0·15% formalin to liquid skim-milk as a means of delaying clotting and permitting consistently sweet milk to be fed under farm conditions to fattening pigs, gave results comparable with those obtained by feeding completely sour skim-milk. This conclusion applied whether the milks were fed in amounts restricted to a maximum of 5¼ pints per pig per day or were given *ad lib.* in conjunction with a daily allowance of 2 lb of meal per pig. There was, in fact, a definite indication that the use of formalin-treated instead of sour milk resulted in slightly less fat being laid down, particularly over the loin, which was reflected in somewhat better commercial grading results. The reasons for this are not clear.

The figures in Table 4 giving the average total consumption of meal + 88% dry matter skim-milk per pig are very similar for pigs on all four treatments, although the pigs given the formalin-treated milk *ad lib.* consumed slightly more meal and less milk than those given sour milk *ad lib.* This difference in total consumption of formalin-treated and of sour milk when fed *ad lib.* is reflected in the average peak daily milk consumption per pig in the two groups. For the pigs given formalin-treated milk *ad lib.*, the average peak daily consumption was approximately 3·1 gal, whereas for those given sour milk *ad lib.*, the corresponding figure was approximately 3·6 gal. For both groups, this peak daily consumption of milk was reached when the pigs were about 19–21 weeks of age, that is 10–12 weeks after the experiment was started.

It would appear, therefore, that when fed *ad lib.* formalin-treated milk was slightly less palatable than the sour milk. In this connexion, it should be borne in mind that the consumption of the formalin-treated milk by the *ad lib.* fed pigs tended to drop slightly when they were first given a freshly delivered batch of milk. This was largely overcome in the latter stages of the experiment, by allowing each batch of formalin-treated milk to stand for 2 days before feeding it.

The closely comparable performance of the pigs fed milk restricted with those fed it *ad lib.* in conjunction with 2 lb of meal per pig per day indicates that this latter system would be a suitable one to adopt should skim-milk ever become available in this country in sufficient quantity and at a low enough price to justify using it as a source of substantial amounts of energy as well as of protein.

In this connexion comparison of the total average consumption of meal and milk of the pigs on treatments (i) and (ii) (restricted milk) with those on treatments (iii) and (iv) (*ad lib.* milk) given in Table 4, shows that by feeding the milk *ad lib.* instead of restricted, in the ways adopted in this experiment, 1 gal of skim-milk replaced 1·1 lb meal. Using prices of £28 per ton for the meal mixture fed and 5*d.* per gal for skim-milk, the total average cost of food per pig fed restricted milk would have been approximately £6. 16*s.* 0*d.* and for a pig fed milk *ad lib.* approximately £8. 3*s.* 0*d.* If the milk was costed at 3¼*d.* per gal, then the corresponding total cost of food would have been approximately the same for both groups, namely about £6. 5*s.* 0*d.* per pig. It is clear, therefore, that using the figure of £28 per ton for the meal mixture, skim-milk would have to be available at 3*d.* per gal or less to make it worth while economically to feed it *ad lib.* instead of restricted to a maximum of 5¼ pints per pig per day. Variations in the current price of the meal mixture would, of course, affect the price at which it would be economic to feed skim-milk *ad lib.* Thus, for skim-milk costing

5*d*. per gal to be economically fed *ad lib*. in the way described, the price of the meal mixture would have to exceed about £47 per ton.

The over-all commercial grading results of the experimental pigs may be considered as average. Previous studies at East Farm (Braude, Mitchell, Cray, Franke & Sedgwick, 1959) have shown the extreme sensitivity, as far as carcass grading is concerned, of the strains of pig at present in the herd, to level of feeding. It was shown, for example, that a reduction of about ½ lb in the maximum daily meal allowance of all-meal-fed pigs from 6 to 5½ lb resulted in appreciably better over-all grading results. The mean figures in Table 4 show that as far as shoulder back fat and loin back fat thickness was concerned, the carcasses had, on average, only a slightly thicker fat layer at these points than the maximum permitted at present to give A gradings at these two points (50 mm for shoulder fat and 30 mm for loin fat). In the light of the previous studies at East Farm on the relation between level of feeding and grading results, it would appear that to improve the back fat grading, a slightly lower allowance of meal than that used in the present trial with restricted amounts of skim-milk, might be given.

The results of the bacon-tasting tests and the absence of any complaints from consumers of the bacon from all the experimental pigs distributed to retailers in the normal way, show that the addition of 0·15 % formalin to the skim-milk, even when fed *ad lib*., had no adverse effect on the quality or taste of the resulting green or smoked bacon.

The experience obtained in the present experiment on the use of formalin in skim-milk indicates that it can be a practical proposition under large-scale commercial conditions. The importance of adding the formalin to the skim-milk as soon as possible after separation, particularly in warm weather, was, however, clearly shown. If the addition of formalin is appreciably delayed, the risk of the treated milk going sour in less than a week, even though a thorough tank-cleaning routine is adopted, appears to be very high.

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The effect of udder infections on the lactation yield of milk and milk solids

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Summary. Data available for fifty cows mainly infected with *Staphylococcus pyogenes* have been analysed to determine the effects of udder infections and mastitis on milk, fat and solids-not-fat yield and on fat and solids-not-fat percentages over full lactations.

It was found that lactation yields of milk, solids-not-fat and fat were depressed by 10, 11 and 12% respectively in the presence of infection. These depressions were significant ($P < 0{\cdot}05$). The composition of the milk averaged over whole lactations was not significantly affected ($P > 0{\cdot}05$), although the mean solids-not-fat percentage of the milk was significantly depressed by $0{\cdot}196 \pm 0{\cdot}092$ during the period of infection.

It is suggested that the depressions in lactation yield and changes in milk composition would have been greater in the absence of antibiotic therapy for mastitis.

Infected quarters, whether showing mastitis symptoms or not, usually yield less milk of lower total solids content than corresponding front or hind quarters of the same udder that are not infected.* Several workers have demonstrated that this reduction in yield and change in composition is very variable (see reviews by Crossman, Dodd, Lee & Neave, 1950; and Rowland, Neave, Dodd & Oliver, 1959) but there is little information on the effect of infection and mastitis on lactation yields of milk and milk solids or on the average fat and solids-not-fat percentages of the milk obtained during the lactation.

Minett & Martin (1936), White, Anderson, Johnson, Plastridge & Weirether (1937), Watts (1951) and McLeod & Wilson (1951) have studied the effect of udder infections on lactation milk yield and have recorded depressions in yield ranging from 5 to 24%. Only White *et al.* (1937) measured the effect on the milk composition over the lactation. They restricted their investigation to the fat content and did not find that infections caused a depression.

The present analysis has been carried out to measure the effect of infections and mastitis on the lactation yields of milk and total solids and on the fat and solids-not-

* In this paper 'infection' means that pathogenic bacteria were recovered frequently from the fresh milk of the affected quarter, and that the milk was positive to the Whiteside test. Mastitis means that the infected quarter showed clinical symptoms of disease.

fat percentages of the milk averaged over complete lactations. The data used were from a herd at the National Institute for Research in Dairying in which the common pathogenic organism was *Staphylococcus pyogenes* and where the cows were promptly treated with penicillin or other antibiotic once mastitis symptoms appeared. Over an 8-year period a complete health history of each quarter of the udders of 240 cows during 530 lactations had been built up in order to investigate the relationship between mastitis and milk secretion and other physiological and environmental factors. The milk of each cow was weighed at each milking, and samples were taken for estimation of the fat content and solids-not-fat content on one day each month. The fat percentage was estimated by the Gerber method, the total solids percentage obtained gravimetrically and the solids-not-fat percentage by difference. Other details of the herd, the management and methods of milking, and the diagnosis of infection have been given in previous papers (Crossman *et al.* 1950; Neave, Dodd & Henriques, 1950; Oliver, Dodd & Neave, 1956).

METHODS

The basis of the analysis is a comparison between the yields and composition of milk secreted in a lactation in which infection and mastitis occur and the previous lactation in which no infection or mastitis occurred. To simplify the presentation of results these will be referred to as infected lactations and infection-free lactations. Because milk yields and milk composition change with age of animal the depressions due to infection and mastitis are calculated from the difference between this first comparison (i.e. between the infection-free and the infected lactations) and a similar analysis of cows with two consecutive lactations free from infection.

To reduce the chances of bias in the results, records were not used if they did not fit certain requirements. Records were excluded if the first of the pair of lactations to be compared was less than 200 days. Infection-free lactations were restricted to those lactations in which infections did not occur and where there was no previous record of infection either in lactation or the dry period. This was because it has been shown that infections either in lactation or dry period can affect the yields in subsequent lactations (Crossman *et al.* 1950; Rowland *et al.* 1959).

After selecting the data in this way records were available from fifty cows with an infection-free lactation followed by an infected lactation, and fifty-four cows with two consecutive infection-free lactations. The validity of the analysis depends on the similarity of the two groups. This can be seen from Table 1 where the relevant information is given.

The average number of infected quarters per cow in the infected group was 1·86 and the average duration of infection 4·5 months. The duration of infection in a lactation was calculated by adding together the number of months each quarter was infected. Details of the type of infection and cases of mastitis are given in Table 2.

RESULTS

The changes in the milk yield were obtained by subtracting the lactation yield of the second of the two lactations from that of the first, for each cow. This was repeated for the lactation yields of fat and solids-not-fat and also for the mean lactation value for fat and solids-not-fat percentage. To investigate the effect on solids-not-fat

percentage independently of variations in the fat percentage of the milk, the mean lactation values for solids-not-fat in the fat-free serum were also compared. In this way two sets of differences were obtained, one between infection-free and infected

Table 1. *Data showing the similarity of the two groups of cows used in the analysis*

	Non-infected cows		Infected cows	
	Infection-free lactation	Infection-free lactation	Infection-free lactation	Infected lactation
Breeds				
Friesian	10		7	
Shorthorn	39		40	
Guernsey	5		3	
Age in lactations, at the 1st lactation of the pair				
First	40	—	34	—
Second	11	—	12	—
Third	3	—	2	—
Fourth	0	—	2	—
Lactation length, days	282 ± 30	266 ± 35	285 ± 25	251 ± 43
Mean calving interval, days	398	384	409	399
Number of cows completing 305-day lactation	22	13	23	8
Number of cows non-pregnant at end of lactation	0	4	0	8
Mean lactation yield				
Milk, gal	707	710	731	658
Fat, lb	266	268	275	241
Solids-not-fat, lb	633	627	655	575
Mean lactation fat percentage	3·8	3·8	3·8	3·7
Mean lactation solids-not-fat percentage	8·98	8·89	8·89	8·71
Number of cows calving in each year				
1945	1		1	
1946	8		2	
1947	15		9	
1948	11		15	
1949	13		13	
1950	14		18	
1951	20		16	
1952	20		17	
1953	6		9	
Number of cows calving in each season				
October to December	19	15	18	17
January to March	17	21	18	16
April to June	4	7	4	9
July to September	14	11	10	8

Table 2. *The lactations in which infections occurred classified according to type of infection and mastitis*

Type of infection	No. of lactations where infections occurred	No. of cases of mastitis
Staphylococcal	27	16
Streptococcal	9	5
Mixed	14	13

lactations and the other between two sets of infection-free lactations, for each of the factors being investigated. The means of these sets of differences and the standard errors of the means are given in Table 3.

It will be seen that the mean lactation milk yield was $73{\cdot}7 \pm 25{\cdot}3$ gal lower in the infected lactations compared with the previous non-infected lactations ($P < 0{\cdot}01$). The difference between the consecutive lactation yields of the control group is much smaller and not significant (i.e. $-2{\cdot}5 \pm 17{\cdot}3$ gal). To estimate the depression due to infection the decline in the control group was subtracted from that of the cows with lactations in which infections occurred. Thus infection and mastitis reduced the yield by $73{\cdot}7 + 2{\cdot}5$ or $76{\cdot}2 \pm 30{\cdot}6$ gal which is significant ($P < 0{\cdot}05$). The depressions due to infections in the lactation yields of fat and solids-not-fat are also significant ($P < 0{\cdot}05$). The declines in the fat and solids-not-fat percentages between the consecutive lactations in the infected group are significant, but when the changes in the control group are taken into account the portions of these declines which can be attributed to infection fail to reach the 5% level of significance.

Table 3. *The mean depressions in the lactation yields of milk, fat and solids-not-fat, and in the fat and solids-not-fat percentages of the milk averaged over lactations*

	Mean depressions			
	Between an infection-free and the following infected lactation	Between consecutive infection-free lactations	Depression due to infection	Percentage depression due to infection
Milk yield, lb	73·7 ±25·3**	−2·5 ±17·3	76·2 ±30·6*	10·0
Fat yield, lb	34·2 ± 9·8**	−2·6 ± 5·2	36·8 ±11·1**	12·4
S.N.F. yield, lb	79·6 ±22·9***	6·5 ±16·8	73·1 ±28·1*	10·7
Fat percentage	0·116± 0·046*	0·009± 0·047	0·107± 0·066	—
S.N.F. percentage	0·179± 0·040***	0·093± 0·024***	0·086± 0·047	—
S.N.F. percentage in the F.F.S.	0·191± 0·038***	0·107± 0·027***	0·084± 0·046	—

* Significant when $P < 0{\cdot}05$. ** Significant when $P < 0{\cdot}01$. *** Significant when $P < 0{\cdot}001$.

It has been shown by a number of workers that as cows grow older the solids-not-fat percentage of their lactation milk yields declines (Bailey, 1952). Although some of this decline might be attributed to the higher incidence of udder infections in older animals it is apparent from Table 3 that there is a highly significant decline in solids-not-fat percentage in successive lactations in the absence of infections.

Contrary to what was expected the control cows did not show a significant increase in yield of milk with advancing age. This is a reflexion of the management of the herd, a factor uniform for the herd as a whole, grouping of data only occurring at the time of analysis.

From Table 1 it will be seen that in both groups the duration of lactation was shorter in the second of the paired lactations. This is to be expected because of the known fall in persistency of lactation with advance in age. It also appears that the infections and mastitis resulted in a greater reduction in duration of lactation; the difference between the decline in the two groups is $16{\cdot}6 \pm 8{\cdot}4$ days which is just significant at the 5% level.

DISCUSSION

In estimating the reduction in lactation milk yield and changes in composition due to udder infections it is necessary to avoid biases caused by the relationship between milk yield and composition, and breed, age, pregnancy, susceptibility to infection, the effect of previous infections and other management factors. This usually means that when the cows are classified into comparable infected and non-infected groups, many have to be discarded, and in fact, in this analysis records of over half of the cows available could not be used. Only McLeod & Wilson (1951) took similar precautions to avoid the possibility of bias in their experimental results. Their comparable analysis gave a depression of 24 % in milk yield which is considerably greater than the 10 % found in this study. The reason for this difference may be that in the N.I.R.D. herd infections were mainly due to *Staphylococcus pyogenes* and cows with mastitis were promptly treated with antibiotic until cured, whereas the infections in the cows used by McLeod & Wilson were caused by *Streptococcus agalactiae* and no antibiotic was used for mastitis cases. Of the fifty infected cows used in the N.I.R.D. analysis thirty-four showed mastitis symptoms (see Table 2) and were treated with antibiotic. Crossman *et al.* (1950) and Rowland *et al.* (1959) have shown that following the elimination of infection from a quarter there is a rapid return to milk of more normal composition, though the recovery is not complete until the following lactation. The milk yield of a quarter does not appear to recover in the same lactation after the elimination of infection but at least it ceases to decline at a greater rate than other non-infected quarters of the same cow. Therefore the treatment of mastitis cases in this study would almost certainly have reduced the depressions due to infection.

The depression due to infection in the solids-not-fat percentage of the lactation yields is not significant at the 5 % level but is significant at the 10 % level. It seems reasonable to believe that this is a real depression because it has already been established that infections depress the solids-not-fat percentage of infected quarters during the period of infection and to some extent after the elimination of the infection. It is to be expected that the effect on the bulk milk will be less because the abnormal milk of the infected quarter will be diluted by varying quantities of normal milk depending on the number of quarters infected and the duration of infection.

To estimate the effect of infections on the solids-not-fat percentage of the bulk milk during the period of infection only, another analysis was carried out. The mean solids-not-fat percentage of the milk of twenty-five of the fifty cows during the period they were infected was compared with the mean solids-not-fat percentage during a similar period of their previous non-infected lactation. The mean solids-not-fat percentages of corresponding periods of infection-free lactations from twenty-five cows were used as controls. This comparison was restricted to the twenty-five of the fifty cows with a lactation in which infection occurred that could be paired with comparable cows in the non-infected group. The animals in each pair were of similar breed and age and calved in the same season of the year. There was a significant depression ($P < 0{\cdot}05$) in solids-not-fat percentage of $0{\cdot}196 \pm 0{\cdot}092$ during the period of infection. Because the average number of quarters infected was 1·9 we can estimate that the depression in solids-not-fat percentage of an infected quarter relative to the non-infected quarters of the cows in this study is nearly double that found by

Rowland *et al.* (1959). There is a possibility that an infection in one quarter may have a depressing effect on the composition of milk from the non-infected quarters of the same udder. This would have the effect of reducing estimates of depression made by quarter comparison. There is no evidence that this occurs and it was not possible to test it in the present analysis.

From the data given in this paper it appears that in a herd in which all cows become infected in two quarters during the lactation, but where mastitis is promptly and adequately treated with antibiotic, the lactation yields of milk fat and solids-not-fat will be considerably depressed, but the depression in fat and solids-not-fat percentages averaged over the lactation will be less than 0·1. In the unlikely event of all cows being infected in two quarters at any one time the solids-not-fat percentage of the bulk milk at that time will be reduced by about 0·2.

We would like to acknowledge the help of numerous people who assisted in the collection of the data used in this analysis. In particular, we are indebted to Dr S. J. Rowland in whose laboratory the chemical analyses of milk were carried out and Dr J. Oliver who carried out the initial tabulation of the mastitis data.

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Reviews of the progress of dairy science

Section C. Dairy Chemistry

THE CHEMISTRY OF BUTTER AND BUTTERFAT

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INTRODUCTION

The task of keeping pace with the literature has necessitated a further division of interests in this series of reviews. In future 'Section C. Dairy Chemistry' will be divided into three or four subsections which will be dealt with in rotation. The first subsection in this arrangement, and the subject of this review, is the chemistry of butter and butterfat. This subject was only selectively treated in the 1958 Dairy Chemistry Review, and with a view to maintaining continuity in the series the literature from early 1955 to the end of 1958 has been covered in the present survey.

This period has been marked by serious problems in the marketing and disposal of surplus production from major butter-producing countries. World butter production increased steadily and reached the highest post-war level in 1957, though, according to the 1958 Dairy Produce Review of the Commonwealth Economic Committee, it was still below the 1938 level (this comparison reflects the marked decline in United States production, from 964,300 tons in 1938 to 638,400 tons in 1958). The world rise in butter production in 1957, not matched by corresponding rises in consumption, led to an increase in world stocks and heavy exports. This culminated in a marked fall in prices on world markets in early 1958 with sharp economic repercussions in the major exporting countries. Reviewing the situation in 1958 the FAO Committee on Commodity Problems took a serious view of the prospects for world trade in butter. It urged measures, both technical and economic, to increase domestic consumption and to place world trade on a more economic footing. Although there was subsequently a rapid change in the world butter position with curtailed production and a sharp upward price movement due to the unfavourable 1959 season in Europe, the long-term problem of disposing of butter surpluses is still evident. In the face of regional food

shortages it represents a major scientific, technical and economic challenge to the world's dairy industry.

One of the highlights of technological progress in the period of this review has been the rapid expansion of the Meleshin continuous buttermaking process in the U.S.S.R. According to Kostygov (1957), Deputy President of the National Committee of Dairying, the number of continuous buttermaking machines in use in Russia rose from 342 in 1955 to 664 in 1956 and to more than 1000 in 1957. The aim is to have 2000 machines in production by 1965. More than 50,000 tons of butter were made in Russia by the continuous process in 1956. Continuous buttermaking has not expanded in Western countries. In the U.S.A. and Canada a recent unofficial report placed the number of Gold'n Flow Machines in use at 30, with six Creamery Package plants. In Australia and New Zealand no continuous process is being commercially used for butter: the Australian New Way continuous buttermaking plant based on the double-centrifuge principle has, ironically, found commercial application only in the manufacture of margarine. Technological progress in the latter countries has mainly centred round the introduction and development of large stainless steel churns up to 100-box ($2\frac{1}{2}$-ton) capacity (Loftus Hills, 1957), mechanical bulk wrapping and packing of butter (Anon. 1957), and the increasing size and complexity of dairy factories (Jackson, 1958).

New books coming within the field of the review included volume II in the series *The Lipids: their Chemistry and Biochemistry* by H. J. Deuel Jnr. (1955). This 919-page volume dealing with the composition and metabolism of lipids in the animal organism has a 20-page section on the synthesis and composition of milk fat. A third and revised edition of the well-known *Textbook of Dairy Chemistry*. Vol. I. *Theoretical* by E. R. Ling (1956*b*) appeared with enlarged sections on the composition and chemical reactions of butterfat. A revised and enlarged third edition of Hilditch's (1956) *Chemical Constitution of Natural Fats* was also published; it has sections on the component fatty acids and glyceride structure of cow milk fat as well as other animal milk fats. Two new text-books on butter by well-known German authors were *Mikrobiologie der Butter* by K. J. Demeter (1956) and *Die Butter* by W. Mohr and K. Koenen (1958), the latter volume of 628 pages covering a wide range of practical and theoretical aspects of buttermaking. Reports of the third All-Union Conference of Dairying held in Moscow in 1953 and the fourth All-Union Conference held in 1956 were published in Russian in book form. The former, of 370 pages, was briefly reviewed by King (1958); the latter contains 90 papers in 472 pages and both volumes have a number of contributions on the chemistry and technology of butter and butterfat. The proceedings of the 14th International Dairy Congress at Rome in 1956 included 53 papers on the technical problems of buttermaking.

Jack & Smith (1956) reviewed the chemistry of milk fat, dealing in particular with the non-glyceride components, the factors influencing the fatty acid composition, the physical structure and the chemical reactions of the glycerides. Shorland & Hansen (1957) dealt with the minor fatty acid constituents occurring to the extent of 2% or less in butterfat. They emphasized recent work on the normal odd-numbered acids and the odd- and even-numbered branched-chain acids, the mechanism of fat formation and details of the isolation and identification of the individual fatty acids in butterfat. Ling (1956*a*), reviewing progress in dairy chemistry, discussed some

aspects of butterfat chemistry. The review of Dastur (1956) on buffaloe's milk and milk products devoted a section to the chemistry and composition of buffalo milk fat.

The present review has been treated as comprehensively as possible within the frameworks of time and subject. Several European journals are not available in Australia, however, and it has been necessary in some cases to rely on published abstracts. Where an article has not been consulted in the original the abstract reference is given in parentheses after the main reference in the bibliography.

BUTTER

(1) *Methods of analysis*

(*a*) *The phosphatase test.* Attention was first drawn to the phenomenon of phosphatase reactivation in HTST-treated cream and butter by Australian and New Zealand workers nearly twenty years ago (Wiley, Newman & Whitehead, 1941). Although this anomalous behaviour of phosphatase has been observed many times since, it is still imperfectly understood. Posthumus (1952) earlier advanced the theory that pasteurization only partly destroys phosphatase present inside the fat globule, this later diffusing to the serum to give a positive test. More recently (Posthumus, 1956) he described further examples of reactivation in butter made from adequately pasteurized cream and observed that the condition was more frequent in winter butter. It did not occur in butter made from milk which was heat-treated before separation. Reactivated phosphatase was more heat sensitive than normal phosphatase as it was destroyed in 5 min at 55 °C. Examples of positive phosphatase tests in pasteurized cream butter due apparently to phosphatase reactivation were also given by Francetic & Jemeric (1957) and Marinelli, Olivo & Venturi (1957).

The official Dutch phosphatase test for butter is based on the Sanders and Sager method; the reaction is carried out in a protein-free filtrate from the butter serum and the results are expressed as μg phenol per gramme of butter. Posthumus & Booy (1958) pointed out a possible discrepancy in this method especially if comparison is made between the phosphatase contents of cream and butter. They observed that when phenol is added to phosphatase-free butter, only about 70 % is recovered in the filtrate used in the official test. They suggested therefore that in expressing the results of the test in terms of phenol content it should be made clear that the figures relate to the phenol content of the filtrate and not of the butter.

(*b*) *Diacetyl.* Brogioni (1954, 1955, 1956) compared three different methods for the determination of diacetyl in butter, based on *p*-toluylenediamine, hydroxylamine and nickel sulphate, and tetra-aminodiphenyl hydrochloride as reagents. The latter method (originally described by Pien, Baisse & Martin in 1937) was found to be the simplest and most sensitive. Values given for diacetyl in Italian butters ranged from 0·25 to 2·10 ppm.

Brandl (1957), after reviewing current methods for determining diacetyl in butter, also chose the method of Pien *et al.* (1937) for further examination. He proposed a number of modifications to the original method, chiefly concerned with the technique of distilling diacetyl from the butter sample, and the method of measuring the yellow colour developed with the tetra-aminodiphenyl reagent. The distillation apparatus was re-designed and steam distillation of 50–100 g of butter in an atmosphere of CO_2

was suggested. With the new apparatus a yield of 99 % of diacetyl from aqueous solution was obtained. The yellow colour from the reaction of diacetyl in the distillate with the reagent was measured spectrophotometrically at 366 mμ. Results by the new technique were reproducible to within ± 1·6 %.

(*c*) *Miscellaneous.* A new technique for assaying the pigments found in commercial butter and margarine was proposed by Espoy & Barnett (1955). In their method the butter is melted and the separated oil dissolved in hexane. This solution can be tested qualitatively by shaking a portion with concentrated HCl; a red colour in the aqueous phase indicates the coal-tar dyes yellow *AB* and *OB*. If the fat solution is shaken with a solution of ammonia in 0·55 % ethyl alcohol, a straw colour in the aqueous phase indicates annatto. In the quantitative analysis carotene is separated from the other pigments by elution from a column of magnesium oxide. The coal-tar colours are extracted from the oil solution with cold 60 % H_2SO_4 from which they are extracted in turn with ethyl ether. Annatto is separated by elution from a sodium carbonate chromatographic column; the three sets of pigments are estimated spectrophotometrically at appropriate wavelengths. Out of forty-seven U.S.A. butters examined by this method thirty-six were found to be artificially coloured, thirty-two with coal-tar dyes and four with annatto.

The official Swedish method for the determination of fat in butter is a modification of the Schmidt-Bondzynski acid extraction technique. Willart (1956) pointed out that results by this method were higher than with the alkaline Röose-Gottlieb technique, the difference corresponding to the amount of free fatty acid present. The disparity was greatest with rancid butter and amounted in one sample to 0·9 % fat. Liberation, by hydrolysis, of 0·008 % fatty acid also occurred with the official Schmidt-Bondzynski method. Gasanova & Kocharyan (1958) suggested a refractometric method for determining the fat content of butter involving solution of a sample in bromonaphthalene; an accuracy of ± 0·2 % was claimed.

(2) *Composition*

Investigations in several countries have been concerned with the increase in curd content and butter yield made possible by eliminating the usual procedure of washing butter at the grain stage. It is now well established that in countries where regulations provide for a maximum moisture content this practice gives an approximate 0·6 % increase in yield.

Inquiries into the phospholipid content of butter and other dairy products have been stimulated by the availability of new techniques for separating this group of compounds.

Aspects of butter composition concerned with fat-soluble compounds (carotene, tocopherol, cholesterol, etc.) are dealt with in the section on butterfat.

(*a*) *Moisture content.* Moisture is not only important in the economy of butter production but its origin, distribution and relationship to other components of the butter system are basic to texture and quality. Mulder (1957) has emphasized the surprising lack of fundamental work on this subject and has discussed some of its theoretical and practical aspects. From a knowledge of the formation and structure of butter he reasoned that the moisture in butter must exist in the following forms: (1) inside the butter granules, (2) on the surface of the granules, (3) in the surface layers

or membranes of the fat globules, (4) in solution in the butterfat, (5) added or 'make-up' water. Mulder discussed the churning and working of butter in the light of this analysis of moisture type and further dealt with the effects on butter moisture of fat content, acidity and heat treatment of the cream, the churning temperature and the speed of the churn, the size and washing of the butter granules, the temperature of the wash water, working and salting.

Moisture losses in commercial butter in cold storage were investigated by Casalis, Serres & Amariglio (1957). The butter was packed in blocks of 25 kg in parchment-lined wooden boxes, stored 6 months at -15 °C and thawed 48 h at ambient temperatures before sampling. Gravimetric analysis of samples did not reveal any loss of moisture through the storage period or any variations attributable to condensation while thawing. Between-sample variation on well-worked butters at the churn did not exceed 0·3 % moisture. In poorly worked butters showing free moisture, variations up to 1·0 % were found. Posthumus & Schuitemaker (1957) were also interested in variations in moisture content of butter at churning. In wooden churns with internal rollers they found deviations from mean moisture values ranging from 0·11 % to more than 0·18 %, with frequent between-sample variations of at least 0·3 %. The best results from the viewpoint of uniformity of moisture distribution were obtained with butter made in metal churns without workers.

(*b*) *Curd or solids-not-fat content.* In a survey of 6801 samples of salted and unsalted butter made in Swedish dairy factories, Bergman & Joost (1956) observed that the solids-not-fat content of salted butter was lower (by 0·09 % in 1954 and 0·03 % in 1955) than that of unsalted butter.

Several reports have dealt with the increased curd content obtained with non-washed butter. White, Beattie & Riel (1956) found the average curd content of 170 churnings of non-washed butter to be 1·31 % compared with 0·78 % for the corresponding washed butters; the average increase in butter yield due to increased curd was 0·53 %. Olsson & Johansson (1957) published interesting data on the effects of size of butter grain, washing and cream dilution on the curd content of sour-cream butter. With a grain size of 10–12 mm and two washings the average curd content for salted butter was 1·12 %; with 16–18 mm grain size and one washing the curd value rose to 1·33 % for salted and 1·53 % for unsalted butter. Increasing the grain size up to 20 mm and eliminating washing gave an average curd content of 2·30 %. Dilution of 36 % fat cream with 3–5 % water lowered the curd content of both washed and non-washed sour-cream butter. Curd contents for butter from undiluted cream were 1·94 % (washed) and 2·38 % (non-washed) while the corresponding values for the diluted cream butters were 1·72 and 2·18 %.

Khotsko (1957) gave some average curd values for Russian butter, 1·63 % for sweet-cream butter by the continuous method, 1·32 % for the corresponding non-washed and 0·84 % for the washed product.

(*c*) *Air content.* Olsson (1956) noted that the machine moulding of bulk butter into 0·5 kg packets reduced the air content by 0·7 ml per 100 g. De Man & Wood (1958) observed much larger reductions in air content (from 3·84 to 1·95 ml/100 g) when bulk butter was moulded. No information was given on the type of equipment used and the conditions for moulding the butter; these would undoubtedly influence the extent of air reduction. The authors described the simple technique used for air determination:

the sample of butter was collected in a brass cone and melted under water at 50 °C, the freed air being collected in a graduated tube. There was a correlation coefficient of −0·94 between the air content and density of the normally churned butters examined. The average gas content of fourteen samples of Gold'n Flow continuously made butter was 0·1 ml/100 g. Winter butter (normally churned) contained 3·4 and summer butter 4·0 ml/100 g.

(*d*) *Copper content.* O'Connor & Grimes (1954–55), concerned about the lack of information on the copper content of Irish creamery butter, examined twelve samples from local factories. The copper contents ranged from 0·03 to 0·80 ppm., six of the samples containing more than 0·2 ppm. The results, they concluded, pointed to the need for a wider use of stainless steel processing equipment.

Swartling (1956) described the occurrence of high copper content (0·10–0·34 ppm.) and inferior keeping quality in the butter from a group of Swedish factories. The contamination was traced to the deposition of copper from wash water on to the surface of equipment. The water, being acid and corrosive, dissolved the copper from the pipes of water heaters. When the water was deacidified, or the copper heating equipment replaced by stainless steel heat exchangers, the copper content of the butter dropped to 0·04–0·06 ppm.

(*e*) *Phospholipids.* Previous studies on the phospholipid content of dairy products have been largely confined to the total phospholipid determination because of the lack of suitable methods for fractionating the individual phospholipid constituents. Baliga & Basu (1955) were the first to use modern biochemical techniques for the routine separation of milk phospholipids into the major lecithin, cephalin and sphingomyelin fractions and later (Baliga & Basu, 1956) they applied their method of phospholipid analysis to butter, cream, ghee and buttermilk. The basis of their method was the adsorption of the cephalin fraction on magnesia and the subsequent estimation of sphingomyelin in the presence of lecithin by selective alkaline hydrolysis. They reported wide variations in the total phospholipid content of creamery butter ranging from 74·85 to 217·56 mg/100 g. The major fraction was cephalin, constituting approximately 45 % of the total phospholipid.

In a short communication Godel' (1957) reported lecithin (presumably total phospholipid) contents of 0·18–0·25 % in normal butter and 0·17–0·29 % in continuous type butter.

McDowell (1958*a–c*) gave the results of a detailed investigation into the phospholipids of butter and other New Zealand dairy products. A modified Röose-Gottlieb technique was used for extracting the phospholipids; lecithin, cephalin and sphingomyelin fractions were estimated from the amounts of choline and phosphorus released by alkaline hydrolysis of the extract. Total phospholipid contents of butter ranged from 0·099 to 0·181 % and the average value 0·139 % was made up of lecithin 0·041 %, cephalin 0·051 % and sphingomyelin 0·047 %. There were marked parallel seasonal variations in phospholipid contents of butter, cream and milk, with maxima in late summer and autumn (February–March) and minima in the winter; lecithin, cephalin and sphingomyelin contents followed the same seasonal trends as total phospholipid. McDowell concluded that the higher phospholipid concentrations in the autumn were due to the smaller average fat globule size in milk from cows late in lactation; with smaller globules the ratio of membrane material, from which the phospholipid is

derived, to fat would be higher. In the third paper of the series (McDowell, 1958*c*) storage at −10 °C for 8 months was reported to have a negligible effect on the total phospholipid and lecithin, cephalin and sphingomyelin contents of salted and unsalted sweet-cream butter. An analysis of serum phosphorus also failed to reveal any evidence of decomposition of phospholipid involving separation of the phosphate group in an acid soluble form.

(3) *Keeping quality*

Investigations in this field have mostly centred around the effects of well-known factors like copper, serum pH, curd content, washing and working practices and storage temperature. The commercial production of synthetic β-carotene has stimulated interest in the effects of butter colours on storage stability. The effectiveness of a number of approved commercial anti-oxidants has invited examination of their behaviour in butter; theoretical aspects of their use in this way have been stressed but there are some potential practical applications.

(*a*) *Copper.* Piraux and co-workers in Belgium (Piraux *et al.* 1956; Piraux, Jamotte, Lheureux & Lacrosse, 1956) and Kruisheer & Krol (1955, 1956) in Holland have given useful information about the effects of a wide range of copper concentration on the stability of unsalted ripened cream butter. Although it is a well-known pro-oxidant, the lowest concentration of copper in butter capable of exerting a harmful effect on keeping quality had not been established previously with any certainty. Piraux and co-workers (1956), summarizing the results of a series of storage trials since 1949, reported that 0·07 ppm. was the upper limit for satisfactory keeping quality and for optimum results the value should be below 0·05 ppm. Where butter of low copper content kept badly it was concluded, from nitroprusside tests, that the heat treatment of the cream had been unsatisfactory. These findings were confirmed by Kruisheer & Krol (1955, 1956) who reported that in summer butter oily and fishy flavours occurred consistently in storage with copper contents of 0·07 ppm. and over, but were absent with values of 0·04 ppm. and less. This relationship did not hold for winter butter in which, despite a consistently higher copper content, there was a relative absence of oxidative defects even with copper levels up to 0·18 ppm. The high winter levels appear to be simply a seasonal variation of the natural copper content; there is evidence therefore that naturally occurring copper in butter does not affect keeping quality. Keestra (1957) also found a high incidence of oxidative rancidity in stored butter with copper contents above 0·10 ppm. but stated that copper levels as high as 0·36 ppm. did not affect the flavour of fresh butter.

Mroczkowski (1957) dealt with the significance of the sources of metal contamination in butter and referred to conflicting reports on this subject. He found that iron had only a weak effect in promoting oxidation whether introduced via the cream or the wash water. The pro-oxidant effect of copper, however, was more pronounced when it was introduced with the cream. According to Koops (1958) butter made from cream of 30–35 % fat content had lower copper contents and better keeping quality than butter made from low-fat (8 %) cream. In a commercial trial in which 0·075 ppm. copper was added to the original milk the high-fat cream yielded butter with 0·059 ppm. copper, while the low-fat cream gave butter with 0·085 ppm.

(*b*) *Serum* pH. As the result of storage studies in Czechoslovakia, Masek, Maxa & Vedlich (1956*b*) confirmed the well-known deleterious effect of acidity on butter

keeping quality and reported that by using incompletely ripened cream with less than 18° SH acidity (0·39 % lactic acid) butter of superior keeping quality was obtained. Piraux *et al.* (1956) also noted the improved keeping quality of cultured-cream butter with serum pH in the 4·8–5·0 range as compared with a pH of 4·5 and lower. Khotsko (1957) also demonstrated the inferior keeping quality of acid salted butter; this was especially noticeable with summer butter which usually became fishy.

It is generally believed that the keeping quality of neutralized-cream butter decreases with increasing serum alkalinity, though this subject does not appear to have previously received detailed study. Surprisingly perhaps, Crittall (1957) reported that in the serum pH range of 6·8–8·2 the more alkaline butters had the better keeping quality. The effect was observed in commercial butters and was confirmed in experimental churnings. In 6 months' storage at 5–10 °F experimental neutral-serum butters lost an average of 3·1 grade points while butters in the 7·3–7·7 pH range lost 2·0 points and at pH 7·8–8·2 the average loss was 1·0 point. There were some departures from commercial practice in the manufacture of these butters; in order to obtain accurate control of serum pH the cream was neutralized to 0·10 % acidity with sodium bicarbonate before pasteurization and adjusted to the alkaline range after pasteurization with 10 N-sodium hydroxide solution. The severe over-neutralization had no significant effect on the initial grade score of the butters but there was an appreciable increase in fat losses in churning the more alkaline creams.

(*c*) *Washing versus non-washing.* In many Australian butter factories in the war and immediate post-war years the practice of washing butter at the grain stage was abandoned. Subsequent experiments in Australia showed that butter quality was not affected and the practice was widely adopted in the southern States. Muller (1955) confirmed that while omission of washing did not affect the keeping quality of well-made butter, under the warmer Queensland conditions churning and manufacturing difficulties were encountered due to the absence of the firming effect of the cold wash water. Where the experimental butters were vacuum-worked and stored in pat form at 45–50 °F a serious flavour defect of unknown cause and described as 'condensed-oxidized' appeared in many of the non-washed butters. It must be assumed that this defect had its origin in some irrelevant factors in the experiment, as many thousands of tons of non-washed butter are now annually vacuum-worked and printed in Australia without difficulty.

The results of comprehensive Canadian experiments with non-washed butter were given in reports by Riel, White & McGugan (1956), White, Riel & Beattie (1956) and White, Riel, Beattie, Smith & McGugan (1957). They support the earlier conclusions that the washing procedure can be omitted without detriment to either the initial quality or keeping quality of the butter. Observations were made on the fat peroxide value, fat acidity, serum pH and copper and iron contents of the experimental butters. The only significant correlations observed were between grade score after storage and copper and iron contents, and between fat peroxide values and serum acidities.

According to data published by Khotsko (1957) in Russia, sweet-cream unwashed butter lost an average of 5·0 grade points after 12 months' storage at −12 °C, compared with 4·7 points drop in grade for the corresponding washed butter. He concluded, however, that the difference was insignificant. Akkerman (1958) also described storage experiments in Russia with washed and non-washed butter. He measured fat

stability after storage by determining its induction period under irradiation from an ultra-violet lamp. After 8 months at −12 °C both sweet- and sour-cream non-washed butter showed less fall in grade and the fat had longer induction periods than the corresponding washed butters. Twice-washed butter was inferior in keeping quality to butter which had a single washing. It was concluded from these studies and from other experiments on the irradiation of milk containing added ascorbic acid that serum constituents exerted a significant anti-oxidant effect in butter.

Where washing of butter is practised, chlorination to relatively high levels may be necessary to control organisms of the genus *Pseudomonas*; dosages of 25 ppm. active chlorine and more have been recommended for this purpose. The effect on butter keeping quality of these levels of chlorine in wash water has not been previously determined and McDowall & McDowell (1956) have described experiments designed to provide information on this subject. Sweet-cream butter was washed during manufacture with water containing up to 75 ppm. active chlorine added as sodium hypochlorite and the butter was stored for 8 months at 14 °F. The use of wash water containing 25 ppm. free chlorine did not affect either the initial quality or the storage quality and up to 50 ppm. chlorine did not cause any increase in the rate of destruction of added diacetyl. At 50 and 75 ppm. chlorine there was a slight lowering of quality in some trials of both the fresh and stored butter. A report from Sweden, however, by Olsson (1957) suggested caution in the chlorination of wash water for acid salted butter; he stated that with a serum pH of 4·7, 5·0 ppm. active chlorine in the wash water led to oily flavours on holding.

(*d*) *The effect of working.* The working of butter under vacuum in Abel-type blenders or wernerizers before printing has been common in Australia for many years (Gunnis, 1958) and vacuum-working during manufacture was investigated in New Zealand in an experimental churn some years ago by Barnicoat (1947). The development in recent years of stainless steel churns capable of operating under vacuum has enabled the commercial exploitation of the possibilities of vacuum-worked butter and a number of reports on the subject have appeared. In general they agree with the earlier observations of Barnicoat (1947) that vacuum-working in the churn has little or no effect on keeping quality of butter though it does modify some of its physical properties. Storgårds & Aule (1956) found that there was no difference in keeping quality between normal and vacuum-worked butter with either ordinary or 'special' (buffered) salting. Swartling, Olsson & Buhrgard (1956) reported a slight but commercially insignificant improvement in keeping quality and lower fat peroxide values with maximum vacuum treatment and prolonged storage. Ystgaard & Korvald (1956) also found that keeping quality was not affected by vacuum-working except for butter with fat iodine values above 36·2 when a slight improvement was observed at intermediate intensities of treatment. Humbert (1956) observed that slightly less oxidation occurred, as judged by the Schibsted fat aldehyde test, in vacuum-worked butter during storage at 40 °F, but there was no improvement in flavour scores. Nagae, Sukegawa & Taneya (1957) described experiments in Japan in which they compared the effects of vacuum-working and working under nitrogen with normally processed butter. They reported that the vacuum treatment gave the best keeping quality.

In a report from Sweden on the relationship between intensity of working and butter quality Malm (1956) stated it was usual, where the churn speed could be varied,

to work butter slowly for a long period and to increase speed towards the end of working. Malm confirmed an earlier Swedish report that while slow working gave a more compact butter with a glossier appearance, keeping quality was adversely affected. With acid salted butter made in rollerless churns a significant lowering of quality was observed after 3 and 6 months' storage in butter worked at 8 rev/min with two ledges, compared with 12 rev/min and four ledges. The loss in quality was associated with higher fat peroxide values. The moisture dispersion in the slowly worked butter was more even but the average droplet size was not affected. No explanation of this interesting phenomenon is offered.

(*e*) *Anti-oxidants.* Studies of the effect on butter quality of commercial anti-oxidants used in the food industry have previously had only a theoretical significance. Some practical commercial possibilities have been opened up, however, by the recent acceptance in Britain of certain specific anti-oxidants in butter for manufacturing purposes.

In an experiment designed to throw light on the origin of the stale condition developing in storage and known as 'storage' flavour, McDowell (1955) incorporated the following known or potential anti-oxidants in New Zealand sweet-cream salted butter: nordihydroguaiaretic acid (NDGA), the octyl and dodecyl gallates, ascorbic acid, citraconic acid, alkali-treated skim-milk, surface-active agents of the Span and Tween series and a number of copper-complexing agents. Most of the compounds tested retarded fat oxidation in storage as indicated by lower peroxide, aldehyde and TBA tests but many gave rise to foreign and chemical off-flavours and none prevented the development of storage flavour. While there are indications that the defect develops in the fat phase and not in the butter serum, its origin remains obscure. An interesting observation which does not appear to have been recorded previously was that the maximum pro-oxidant effect of salt occurred at the intermediate concentration of 1·6%. Increasing the salt content to 2·5% decreased the extent of fat oxidation to little more than that in unsalted butter.

The improvement of butter keeping quality by the use of yeast cultures has been reported from time to time by Russian workers. Masek, Maxa & Vedlich (1956*a*, *b*) gave a summary of experiments with selected strains of Torulopsis yeasts which were worked, in a water suspension, into sweet-cream and sour-cream butter. They reported a beneficial effect on keeping quality in both types of butter presumably due to the production by the yeasts of reducing substances and the reduction of serum acidity. The yeast cultures were very little inferior in effect to a patented anti-oxidant preparation 'Laktokys' concerning which no details were given except that it contained lactic acid as a major ingredient. Cerutti (1956) found that, of the four anti-oxidants ascorbyl palmitate, ascorbic acid, propyl gallate and butyl hydroxyanisole, the former gave the best keeping quality and lowest peroxide values in butter after 150 days' storage at 0 °C. Novotel'nov, Grishchenko & Abramova (1955) described the anti-oxidant properties in butter of various products obtained from the enzymic digestion of the dog-rose fruit.

Sedlacek, Rybin & Ticha (1957) stored samples of butter and lard for 5 months at 5 °C in parchment wrappers treated with quercetin, quercetin and citric acid, and dihydroquercetin. They reported that oxidative rancidity, judged by organoleptic and chemical tests, was slowed down even in the interior of the samples with the

treated wrappers. The influence of similarly treated parchment wrappers and aluminium foil wrappers on oxidase activity in stored butter was determined by Hrabakova-Zvonickova (1957) using a 1-naphtholdiethyl-*p*-phenylenediamine reagent; no effect was observed.

(*f*) *Carotene and vitamin A*. The successful commercial production of synthetic β-carotene in 1954 by Hoffman-La Roche provided an ideal medium for artificially colouring butter, and a number of papers have appeared dealing with the influence of the synthetic compound on butter quality. No adverse effects on either the flavour or keeping quality of butter have been attributed to synthetic β-carotene itself though some technical difficulties, such as the question of a suitable vehicle, have been experienced. Aust (1955) reported favourably on β-carotene for colouring butter. The colour was dissolved in an edible oil and added to the cream before churning and the butter was stored at −2, 5 and 10 °C. The flavour, keeping quality and fat peroxide values were not affected. Stüssi (1957) found that while synthetic β-carotene added directly to butter improved keeping quality and lessened fat oxidation, incorporation of oils used as vehicles for the colour lowered flavour scores. Some samples of sesame oil gave best results for this purpose. Butter coloured with annatto kept better than butter containing added β-carotene for a short period in cold storage but over an extended period β-carotene gave best results. In Canada, Riel (1957) advocated the replacement of coal-tar dyes for colouring butter with synthetic β-carotene following an investigation in which satisfactory results were obtained with the new colours (Riel & Johns, 1957). A 'coal-tar' dye and synthetic β-carotene were compared in neutralized-cream butter at low quality levels and in the presence of added copper. The β-carotene was added to the cream as a 0·5 % paraffin-oil suspension. The type of colour did not affect the flavour scores after storage up to 6 months at −5 °F and no significant differences were observed in fat peroxide and aldehyde values and acid degree. Riel (1957) observed that while the colouring of Canadian winter butter with β-carotene did not increase the vitamin A potency to that of summer butter, a minimum level of 12,000 i.u. per lb could probably be maintained. In a further investigation on butter colours Riel & Gibson (1958) compared the effects of annatto and coal-tar dyes on butter keeping quality; they observed no difference either in grade scores or tests for fat oxidation between the two types of colour.

The role of β-carotene in the oxidative deterioration of butter has not been established with any certainty. Schuller (1957) determined the effect of carotene alone and in combination with diacetyl, ascorbic acid, lecithin (the source of this compound is not given) and copper on butter deterioration. A 'model' butter system was used; butterfat was freed from carotene by treatment with activated carbon and known amounts of synthetic β-carotene were added back. The fat was emulsified under nitrogen with water and cooled and worked to butter with a specially designed worker. Oxidation was followed at 14 °C by peroxide tests. Carotene behaved initially as an anti-oxidant, the degree of protection varying directly with the concentration. This early induction period was shortened by exposure of the samples to light or by the additives studied, and was followed by pro-oxidant activity. Schuller concluded that decomposition of carotene protected the fat in the early stages and that possibly breakdown products accelerated fat oxidation after this period. Poryadkova (1957) also concluded that the vitamin A activity of butter, including carotene, was a factor

in storage stability. He reported that sweet-cream butter made from milk rich in total vitamin A kept better in storage than butter low in vitamin A, particularly as judged by serum acidity and amino-nitrogen values, fat acidity and ease of fat oxidation after storage. Differences in flavour quality were not marked but appeared to favour the vitamin A-rich butters.

(*g*) *Temperature of storage.* Increased use of stored butter in Australia for domestic consumption has focused attention on storage problems there. Pont & Gunnis (1958) made a detailed study of the effect of storage temperature on the keeping quality of over 2000 boxes of sweet-cream and neutralized-cream salted butter in Melbourne and Brisbane and advised that no improvement in quality could be expected by lowering temperatures below the level of 12 °F in current use. They found only insignificant differences, both in interior and surface quality, over the temperature range −10, 0 and 12 °F after 3 and 6 months' storage. There were indications also that the lower temperatures favoured cracking of the butter.

(*h*) *The effect of light.* Better industrial lighting and modern display methods of selling constitute a possible hazard to butter quality through excessive light exposure. Malm & Hildingson (1955) drew attention to the ineffectiveness of conventional parchment wrappers in protecting salted ripened cream butter from light in illuminated cold display cabinets. Deterioration in quality after holding 10 days was evident, from flavour scores and fat peroxide tests, even with exposures to fluorescent globes as short as 1 h. With short exposures fluorescent light was more damaging than light from filament lamps, but with exposures of 4 h or more there was little difference. Aluminium foil wrappers gave effective protection against light. Aigner & Aust (1955) irradiated sweet-cream butter with fluorescent lamps using different types of yellow filters to cut off the near ultra-violet light. They found a loss in keeping quality in all the irradiated samples, the deterioration being least with a yellow glass for the fluorescent tube itself; the use of similar globes was advocated in butter factories and stores.

(*i*) *Continuous versus batch manufacture.* The results of several Russian investigations involving comparisons between the quality of continuously made and conventionally churned butter have been published. The results have generally favoured the continuous method. Khotsko (1957) observed a loss of 4·0 grade points in sweet-cream continuous butter after 12 months at −12 °C compared with 4·7 points loss for churned washed butter and 5·0 points for churned non-washed butter. The superiority of the continuous butter was more marked when held at above zero temperatures. No differences were found between wooden and metal rollerless churns in their effect on keeping quality. Vyshemirskii (1958) compared the keeping quality of commercial continuously made and normally churned butter from each of several factories in five different areas in Russia; the butters were held at −6 to −16 °C for 12 months. The average drop in grade was 4·5 points for the churned and 3·8 points for the continuous butter. The fat acidities throughout were similar but the increase in serum acidity of the normal butter during storage was twice that of the continuous type. According to Lapshina (1958) continuously made sweet-cream butter showed no seasonal differences in storage stability at −3 to −10 °C. Continuous acid winter butter, however, whether salted or unsalted, kept much better than did the corresponding summer butter which developed fishy and oily flavours.

(4) *General*

(*a*) *Quality testing.* Many laboratory tests have proved useful in the diagnosis and control of quality defects in butter. In view of the complexity of the chemical and biological factors concerned, however, it is not surprising that the correlation of any particular test result with quality has been found to be poor and the use of such tests for predicting keeping quality has been unsatisfactory. Ommodt & Hendrick (1956) gave the details of an analytical and quality study of 133 samples of commercial U.S.A. butter in the three quality grades AA, A and B. As the authors were interested in the immediate marketing value of the butters, keeping quality was judged by holding 7 days at 21 °C, followed by 30 days at 2–7 °C. Although copper contents averaged from 0·197 to 0·255 ppm. over the three grades and ranged up to 5·15 ppm. in individual samples, it was concluded that copper (and iron) contamination was not serious. (While these copper levels may have been mostly without effect on the short-range keeping quality of the butter at above freezing temperatures, they would undoubtedly seriously affect long-term keeping quality in cold storage.) Ommodt & Hendrick failed to observe any correlation between keeping quality and the results of any single bacteriological or chemical test. Schulz & Kley (1956) also gave a detailed account of a series of chemical, physical, microbiological and short-term keeping quality tests carried out on German sweet-cream and sour-cream butters. The keeping quality was determined on the basis of 3 weeks' storage at 15 °C. The authors aimed at a basic understanding of the results of sensory testing, and eventually the establishment of analytical criteria for the marketing quality of butter. An evaporation test for moisture dispersion indicated many poor samples and this particular test appeared worthy of further development. However, from a study of the relationship between the results of the various tests, the initial quality and the keeping quality of the samples, Schulz and Kley concluded that the marketing quality of the butter could not be measured in this way unless new and better tests were developed. In a further examination of the evaporation test (Schulz & Voss, 1957) it was concluded that though poor quality butter had a high evaporation rate, the quality could not be judged by this test alone.

A brief report on a study in Bulgaria by Todorov (1956) states that acidity, peroxide, reductase and fermentation tests were of some value in assessing the keeping quality of butter, though the results should only be accepted as tentative. Sode-Mogensen (1957) gave a detailed survey of a wide variety of chemical, physical and bacteriological tests used for butter quality control in Denmark.

(*b*) *Canned butter.* Dissatisfied with the keeping quality of normal canned butter above 7 °C, Hansen, Wood & Thornton (1956) developed a process for canning butter under nitrogen followed by an in-can heat treatment at 77–82 °C. The liquid mixture was emulsified and solidified by shaking at −20 °C. They claimed that the product had a satisfactory texture above 7 °C and had excellent keeping quality, no flavour defects being evident after 6 months at 20–32 °C. The butter was still acceptable after 19 months at these temperatures with no increases in peroxide and TBA values and only slight increases in fat acid values. A rather similar in-can heat treatment for canned butter was described by van den Berg (1957); the treated butter was reported to keep for 6 months at 25 °C, but with some development of chemical off-flavours.

In a later report Hansen, Wood & Thornton (1957) described the properties of a canned butter concentrate, prepared on a laboratory and pilot plant scale from butterfat, skim-milk powder, salt, NDGA and a small proportion of hardened peanut oil. The samples were examined in comparison with a commercially prepared Australian product of similar composition. The flavour stability in these products was of a high order, most being of acceptable quality after 24 months at 32 °C, though it was reported that samples with added diacetyl became tallowy within 4–12 months irrespective of temperature (this is not in accordance with Australian experience with this product). Iodometric peroxide and TBA values generally were very low throughout the tests and only slight increases in fat acidity were observed. A brief report is also at hand from Russia on the keeping quality of a type of butter concentrate. Chinenova & Minkvits (1957) gave results of storage tests up to 15 months with a low moisture (1·6 %) 'butter'. Good stability was claimed on the basis of fat acid, peroxide and aldehyde tests.

(*c*) *Miscellaneous.* Thomsen (1956) discussed the chemical and physical properties of whey-cream butter and described experiments aimed at improving handling and manufacturing procedures for this product. The inferior keeping quality of whey butter was attributed to its lower serum solids, a lower content and altered distribution of phospholipids and heavier metal contamination. Only about 9 % of the fat in whey butter has been found to be in the globular form and the frequent leaky body and poor spreadability of whey butter is probably due to the abnormally high free-fat content. Slow cooling of whey cream after pasteurization, small grain size, prolonged chilling of grains with wash water (10–15 min at 37–43 °F) and thorough working were recommended to control texture defects.

Popiel & Broniszewska (1958) described experiments in Poland with chemical 'starters' instead of normal souring of cream for buttermaking. The chemical solutions used, which were reported to contain 30–50 % lactic acid and 0·10–0·21 % diacetyl, were added either at the grain stage or during working of the butter. The over-all quality of butter prepared in this way from sweet cream was equal to that of normal ripened cream butter and it was reported to be superior in keeping quality.

BUTTERFAT

(1) *Composition*

(*a*) *Fatty acid composition.* The discovery of branched-chain fatty acids in butterfat in 1951 and *n*-odd-numbered fatty acids in 1954 by Shorland, Hansen and co-workers opened new chapters in the chemistry of butterfat composition and formation. This work has been continued and amplified with the reported isolation of the *n*-C 15 and *n*-C 13 acids by Shorland, Gerson & Hansen (1955*a*, *b*) and the *n*-C 17 acid (margaric acid) by Hansen, Shorland & Cooke (1957). Gaps in the series of known branched-chain acids in butterfat were filled with the discovery of the C 13 and C 15 *iso* and *ante-iso* methyl-branched-chain acids (Shorland *et al.* 1955*a*, *b*). James & Martin (1956) reported a wide range of *n*-odd acids in goat's milk fat, and Shorland & Hansen (1957) estimated that these acids constitute about 2 % of the total weight of fatty acids in butterfat with probably a complete odd and even range from acetic to cerotic acid.

Using gas chromatography, Hawke (1957), however, failed to detect volatile odd C acids in fresh butterfat but isolated formic, propionic, valeric and nonanoic acids from oxidized butterfat. He cautioned against confusing fatty acids formed by secondary processes with those occurring naturally. His observations may have some bearing on the report from Corrao (1957) that formic and acetic acids were detected by paper chromatography in distillates containing volatile acids from butterfat. More detailed information on recent progress in the chemistry of the minor fatty acids in butterfat will be found in the review by Shorland & Hansen (1957).

Garton & Duncan (1956), using an ester-fractionation technique, investigated the fatty acid composition of milk fat from stall-fed beef cattle in relation to diet. They observed that fat from cows fed on roots was much less unsaturated than that from silage feeding and that there appeared to be a reciprocal relationship between the content of oleic acid and palmitic acid, these two acids accounting for 53·0–56·8 % of the total. They concluded that the degree of fat saturation was less significant than the tocopherol content in the etiology of muscular dystrophy in suckling beef cattle. Garton, Duncan & Lough (1957) later described the effect of drenching cattle with tung-oil on the fatty acid composition of the milk fat; with 100 g of the oil daily for 6 days the conjugated trienoic fatty acid content was raised from 0·06 % to values from 0·18 to 0·33 % and the conjugated dienoic content from 0·7 to 1·6–7·4 %.

Tawde & Magar (1957*b*), observing that little information was available on the fatty acid composition of Indian butterfat, presented the results of an analysis of three samples of cow ghee. Saturated fatty acids over the range C4 to C20 comprised 63–64 % of the total, the principal one being palmitic acid. The chief unsaturated acid was oleic; the composition of the samples generally was within the range of reported values.

Subbaram & Mahadevan (1957) examined iso-oleic acid isolated by the Bertram method from beef-body and cow-butter fat. By the technique of oxidation and separation of the resultant dibasic fatty acids by partition chromatography they confirmed earlier observations that vaccenic acid obtained from these sources is not homogeneous; they found the material from cow butter to consist of Δ^{13}-octadecenoic acid 20·5 %, Δ^{11}-octadecenoic acid 49·3 % and Δ^{9}-octadecenoic acid 17·4 %. Lack of homogeneity in oleic acid isolated by the usual means from butterfat was also the subject of an investigation by Backderf (1956). He separated the C18 acids from butterfat as methyl esters, first by fractional distillation and finally by crystallization from methanol. The techniques of oxidative cleavage and infra-red analysis were used to determine the double bond positions and configurations. The following acids were identified: *trans*-11-, *cis*-11- and *trans*-16-octadecenoic (the latter had not been previously reported as naturally occurring), *cis*-9- and *cis*-12-octadienoic, and various C16 acids mostly with the *trans*-configuration. *Trans*-11-octadecenoic (vaccenic) acid was believed to constitute 3–4 %, and the new acid, *trans*-16-octadecenoic, 1–2 % of the total fatty acids in butterfat.

Scott, Herb, Magidman & Riemenschneider (1957) also reported briefly the results of investigations into the *cis-trans*-configurations of unsaturated fatty acids in fractions prepared from butterfat by a variety of techniques. Their data indicated the presence of monoethenoid *cis*- C10 to C14 and *cis*- and *trans*- C16 and C18 acids,

cis-trans and *trans-trans* conjugated C18 acids, and *cis-cis*, *cis-trans* and *trans-trans* non-conjugated diethenoid C18 acids. Only the *cis-* form of trienoic, tetraenoic and pentaenoic acids was observed.

(*b*) *Non-glyceride constituents.* The recent literature on the vitamin A, carotene and tocopherol content of butter was reviewed by McGillivray (1958) in the Biennial Review of that year dealing with the nutritive value of milk and milk products. McGillivray summarized a larger number of reports on the vitamin A and carotene contents of butterfat from geographically diverse areas and commented that the results were in substantial agreement with those reported earlier, and that the seasonal variations observed could in most cases be associated with periods of stall and pasture feeding. He drew attention, however, to the position in New Zealand where appreciable seasonal variations in vitamin potency occur in spite of the availability of pasture throughout the year; interference by unknown factors in the utilization of plant carotene can be suspected. The reader is referred to McGillivray's review for more details, and the appropriate references on the above subjects.

Later references to the vitamin A and carotene content of butter were those of Khotsko (1958) and Ermakova (1958), both workers reporting the vitamin A potency of Russian summer butter to be three to four times that of winter butter. Khotsko (1958) stated that ripened cream butter had lower initial vitamin A activity and greater storage losses than the corresponding sweet-cream butter. Radema (1958) also observed an approximate threefold increase in carotene content of butterfat in Holland on change to pasture feeding.

Lobanov, Vasil'eva & Gorelova (1958) gave the results of cholesterol determinations in various dairy products; the values for butter ranged from 0·231 to 0·239 %.

(*c*) *Seasonal and regional effects.* Numerous papers have been published dealing with the effect of season and other influences on the composition of butterfat in different regions. In some the emphasis has been on the limits of variation in the classical chemical and physical constants and certain of their interrelationships chiefly as criteria of adulteration, in others the aim has been the exploitation of new techniques of analysis and a better understanding of the physical and chemical properties of butter and butterfat. Results of investigations and surveys in these fields were given by Keeney (1956) and Zehren & Jackson (1956) in the U.S.A., Kehar, Ray, Joshi & Raisarkar (1956) in India, Kuzdzal-Savoie (1956*a–c*, 1957) in France, Nagae *et al.* (1958) in Japan, Reinart & Nesbitt (1956) in the Manitoba Province of Canada, Riel (1955, 1956) in Canada, Rutz, Martin & Whitnah (1955) in Kansas, U.S.A., Sampath & Anantakrishnan (1956) in India, Shorland & Hansen (1956) in New Zealand, Stadhouders & Mulder (1955, 1956) in Holland, Steen (1956*a*, *b*) in Denmark, Naudts & de Vleeschauwer (1956) in Belgium, Olivari (1956) in Italy, and Wood (1956), Wood & Haab (1957), Haab (1958) in Alberta, Canada. The results from the northern hemisphere are on the whole consistent with the well-known seasonal trends in that region with maximum volatile acid contents in the spring and summer and maximum iodine values in the summer and autumn. This is in sharp contrast to the pattern in New Zealand where maximum iodine values and oleic acid contents of butterfat occur in the winter. Shorland & Hansen (1956) remarked that the trends in New Zealand were consistent with modern concepts of fatty acid formation in that high contents of

short-chain acids occur in the spring period of maximum pasture intake. Similarly, a high content of oleic acid, derived from body fat, is associated with the winter period when the plane of nutrition of cows on pasture in New Zealand is lowest. (Mayhead & Barnicoat (1956), however, concluded from feeding experiments with cows in New Zealand pastures that the rise in iodine value which follows partial inanition does not result from utilization of body reserve fats.) In the relationship between fatty acid composition of butterfat and season in the northern hemisphere the necessity for winter stall feeding is, no doubt, a complicating factor. Various aspects of this subject are also discussed by Hilditch (1956).

(*d*) *Other factors influencing composition.* According to Beninati & Valenti (1956) Reichert-Meissl values of milk fat from cows which had suffered stress (hunger, thirst, transport, etc.) ranged from 19·2 to 21·96, compared with an average value of 27·24 in fat from normal market milk. The effect appears to be the well known one of inanition and consequent lack of acetate for fatty acid synthesis.

Lagoni & Samhammer (1956) found a high inverse correlation ($-0{\cdot}84$) between the consistency of butter and its iodine number over a wide range of values (28·3–41·1). The relationship was evident even after reworking. Orth & Kaufmann (1957) investigated the connexion between the consistency of milk fat and the unsaturated fatty acid and crude fibre content of the fodder. They observed relationships between the linoleic and linolenic acid content of the fodder, the milk fat and the consistency of the butter. Mayhead & Barnicoat (1956) observed the effects of a number of factors on the iodine value of the milk fat of cows maintained on typical New Zealand pasture. The average iodine value of the milk fat of a group of five Jersey cows through the 14 months' testing period was 2·2 units lower than that of a group of Friesians, though there were large individual within-breed variations. Both groups showed the same seasonal trend with maximum iodine values in the winter. The morning values averaged about 1·8 units less than the evening values.

Following successful New Zealand experiments on the control of bloat by treatment of pastures or by drenching with anti-foaming agents, several papers have dealt with the effect of treatments on the properties of the milk fat. McDowall, Patchell & Reid (1957) considered whale oil was unsuitable for this use as it had marked effects on the flavour of the cream and butter and on the properties of the fat. With a drench of 300 ml whale oil per day for 6 days the fat iodine value rose from 30 to 60 and the softening point fell from 33·5 to 27·5 °C. Drenching with linseed, soybean or peanut oil gave better results, but at the higher dosage levels substantial changes in fat constants, particularly iodine values, were still evident (McDowall, Reid & Patchell, 1957). The use of a tallow emulsion at the rate of 125 g fat equivalent twice daily had only minor effects on butterfat properties and was recommended for bloat control if technical difficulties in use could be overcome. Administration of paraffin oil (McDowall, McGillivray & Reid, 1957; McGillivray, 1957) had no effect on butterfat composition but severely depressed vitamin A and carotene levels.

Kniga and co-workers (Kniga, 1958; Kniga, Babak & Khmelik, 1958) examined the physical and chemical properties of butterfat in relation to the carbohydrate and fat levels in the fodder. They stated that previous experiments had shown that butterfat with the best technological properties had a 1·7 to 1 ratio of non-volatile saturated to unsaturated fatty acids, an iodine value of 31 to 34, and a Reichert number of 31 to 32.

Such a fat was obtained in controlled feeding experiments when the fodder ration contained not less than 100–120 g sugar per kg of milk and 60–70 g of fat per 100 g of milk fat.

(2) *Methods of analysis. Adulteration*

The search for new and improved methods for the detection of foreign fats in dairy products continues to provide one of the most active fields of research in butterfat chemistry. New developments in fat technology and the growing commercial exploitation of 'filled' dairy products make the task of distinguishing between pure and adulterated butterfat increasingly difficult and urgent. Judging by the geographical distribution of inquiries the problem of detecting butterfat adulteration appears to be most acute in North America, Italy and India. The subject has been reviewed most recently by Bhalerao & Kummerow (1956*b*) and it was discussed by Aschaffenburg (1958) in the last Dairy Chemistry review of this series. Bhalerao & Kummerow (1956*b*) indicated the limitations of various tests used or proposed for the detection of foreign fats and emphasized the complexity of the problem in view of the increasing variety of synthetic and modified fats available. The most useful test they found for detecting 10 % admixtures of a range of substitute fats was one based on glyceride structure and developed by themselves (Bhalerao & Kummerow, 1956*a*).

(*a*) *Tests based on volatile fatty acids.* Methods of detecting adulteration based on butyric acid and the volatile fatty acids are becoming increasingly uncertain in the face of new commercial techniques for rearranging and modifying edible fats. Bhalerao & Kummerow (1956*a*) pointed out that it was possible to prepare an imitation butterfat from a mixture of cheap fats like beef-fat, tributyrinated lard and coconut oil which would defy analytical detection especially by techniques based on butyric acid content. Murthy (1955) discussed the limitations of the Reichert-Meissl determination as a measure of adulteration and concluded that it was impractical to judge the purity of butterfat on the Reichert-Meissl value alone.

Anglin & Mahon (1956) gave the results of collaborative studies on a modified and more reproducible Keeney chromatographic technique for determining the molar percentage of butyric acid in butterfat. The ranges of Grossfeld butyric acid number and Wollny, Polenske, Zeiss and Tortelli-Jaffe values for forty samples of Italian butter and various mixtures with substitute fats were reported by Cerutti & Tamborini (1956*b*).

Shipe (1955*c*) and Bassette & Keeney (1956*a*, *b*) presented improved hydroxamic-acid-ferric chloride colour tests for characterizing butterfat. The latter authors reported that the results of the test were closely related to the butyric acid content and they believed the method should be useful for detecting 20 % or more of foreign fat.

Curli (1955) suggested a new analytical method for detecting adulteration by comparing the ratio between two fractions obtained in the Reichert distillation. Antoniani & Daghetta (1958) reported favourably on the method. A test involving separation and comparison of fatty acid fractions from hydrolysed butterfat was also proposed by Mecca & Pogliano (1958).

(*b*) *Tests based on* iso-*valeric acid.* Aschaffenburg, in the 1958 Biennial Review, remarked that tests based on the detection of *iso*-valeric acid were emerging as the solution to the problem of dolphin-oil adulteration of butter in Italy and subsequent

references confirm this conclusion. Cerutti & Tamborini (1956*a*), in presenting the results of a large number of normal and adulterated Italian butters, indicated that sure detection by chromatographic isolation of *iso*-valeric acid had reduced the use of dolphin oil as an adulterant though whale oil and lard were being increasingly used. Priori (1955) and Canuti (1958) described paper-chromatographic techniques for the identification of *iso*-valeric acid in butter and both claimed that 5 % adulteration with dolphin oil was detectable by these methods. Chioffi (1956) developed a technique for detecting acetic and *iso*-valeric acids in butter based on their partition coefficients between water and CCl_4 by which they can be distinguished from the natural volatile acids. The detection of 10 % extraneous fat in butter was claimed.

(*c*) *Urea-fractionation methods.* Detection of butterfat adulteration by precipitation of fatty acids as urea complexes was first suggested by Holasek & Ibrahim (1953). Shipe (1955*a*, *b*) suggested for the purpose a urea-fractionation method including determination of the refractive indices of the various fractions and found it more sensitive than the hydroxamate test. He emphasized the highly empirical nature of the procedure however, and Bhalerao & Kummerow (1956*b*) believed it would be difficult to detect coconut oil or synthetic fat mixtures with the method. More recent studies with urea-fractionation methods have not answered these objections. Tawde & Magar (1957*a*) believed that urea-fractionation should be especially useful in distinguishing hydrogenated fats as it complexes more readily with *cis*- than with *trans*-isomers. By using the technique in combination with physical examination of the urea-precipitated fractions they detected adulteration of ghee with vanaspati down to the 5 % level. Sengupta, Shipe & Dahlberg (1958) proposed a complex systematic examination of butterfat for adulteration combining Reichert-Meissl and Polenske determinations and urea fractionation of the non-volatile acids.

(*d*) *Tests based on non-saponifiable constituents.* Anglin, Mahon & Chapman (1955) and Mahon, Anglin & Chapman (1955) reported on the use of a rapid tocopherol test for detecting adulteration of butter and other dairy products in Canada. They gave the limits of seasonal variation in tocopherol content found in 329 genuine samples of butterfat (Canadian regulations now stipulate a maximum tocopherol content of 50 ppm.) and stated that tocopherol contents as high as 480 and 168 ppm. had been found in adulterated fats from commercial butters and ice cream respectively. They agreed, however, that the test, while useful for detecting most vegetable oils, would fail with coconut oil. Modifications and improvements to the Mahon and Chapman test for tocopherol in butterfat were described by Windham (1957). As the natural tocopherol content of samples of ghee was found by Ramanujan & Anantakrishnan (1958) to range from 20·6 to 44·5 ppm. they concluded that adulteration with less than 10 % of peanut oil could not be reliably detected by tocopherol analysis.

Differences in physical properties of plant and animal sterols and particularly in the melting-points of certain of their derivatives have long been used to distinguish animal and vegetable fats. Such tests have similar limitations to the tocopherol test in that they cannot detect admixtures with animal fats and are insensitive to coconut oil. Cannon (1955, 1956, 1957, 1958) has reported continued refinements and improvements to the sterol acetate test in butterfat.

(*e*) *Other tests for adulteration of butterfat.* The Tortelli-Jaffe test, based on a colour reaction with bromine, was first used 40 years ago to distinguish between marine and

land fats. The simplicity of the test has awakened interest in its possibilities for detecting adulteration of butterfat but the results appear to be inconclusive. Cerutti (1955*a–c*) refined the test by the use of chromatography and claimed it could detect 5% of marine fat in butter as well as small admixtures of sheep butter. Olivari (1955), however, after examination of genuine and adulterated samples with the method, concluded that it was only useful in combination with other tests.

Krienke (1955) described an improved fat fractionation technique for detecting adulteration which was sensitive to 3% of foreign fat in butterfat. Turbidity-temperature techniques, using acetic acid as a solvent, were suggested by Mahon & Chapman (1955) and benzyl alcohol-glycerol by Desikachar, Patwardhan, Sastry, Srinivasan & Subrahmanyan (1957), the latter authors being concerned with the adulteration of ghee with vanaspati. Narayanan & Kartha (1955) proposed the determination of *iso*-oleic acid, and Chatterji & Chandra (1957) differences in molecular refraction as indices of this type of adulteration. Venkatachalam & Sundaram (1957) described a turbidity technique by which they were able to detect 1% of mineral oil in ghee. Sesame oil has been suggested as an indicator to be added to hydrogenated fats in India but Chakravarti & Dash (1956) considered it was unsuitable and suggested a more stable and specific substance like quinine.

Armandola (1956) discussed the causes of variation of physico-chemical constants of butterfat. Vitagliano & d'Ambrosio (1957) also dealt with legal standards for butterfat composition in Italy and discussed, in the light of their own experiments, their usefulness as criteria of purity.

(3) *Oxidation and other deteriorative reactions*

Although development of the techniques of chromatography and spectrophotometry has led to recent advances in the study of lipid oxidation, many important problems in the oxidative deterioration of butter and other dairy products await solution. In particular the relative importance of triglyceride and phospholipid oxidation and their respective contributions to flavour defects arising in cold-stored butter are yet to be clarified. Judging by the output of papers interest in this field appears to have recently declined slightly. Many of the latest investigations have relied on the techniques of column and paper chromatography for the isolation and separation of compounds resulting from fat oxidation. Few of these studies have been conclusive and it seems likely that the possibilities of these techniques have been exhausted for this work. The further refinements of gas chromatography offer new prospects for progress, and results from the application of this technique to the identification of the specific substances responsible for oxidized flavour defects in butterfat are awaited with interest.

(*a*) *Oxidation of triglycerides.* Hammond & Bird (1955), in an examination of oxidized butterfat, applied the now well-known techniques of separating carbonyl compounds with Girard's reagent and fractionating their 2,4-dinitrophenylhydrazones, first on the basis of solubility and secondly by column chromatography. Their work, however, was described only briefly. Tamsma (1955*a*) sought to identify the compounds responsible for the characteristic flavour of oxidized milk fat by solvent extraction of steam distillates from the fat, followed by chromatography on a

variety of adsorbents. In a subsequent paper Tamsma (1955*b*) described a procedure for separating carbonyl compounds from the distillates by chromatography on Celite combined with formation and crystallization of the semi-carbazide derivatives. He agreed with other contemporary workers in concluding that the compounds responsible for oxidized flavour in fat were essentially carbonyl and unsaturated and were probably ketonic.

Vachha, Leley, Narayana & Daji (1957) followed the oxidation of cow and buffalo ghee at 98 °C by measuring the rates of uptake of O_2 and evolution of water and CO_2. They commented that the absorption of O_2 followed the sigmoid curve characteristic of an autocatalytic reaction. Buffalo ghee oxidized at about twice the rate of cow ghee; this was attributed to the anti-oxidant effect of the much higher carotene content of the cow ghee. Vachha *et al.* observed that appreciable quantities of water and CO_2 were evolved from the beginning though during the first few hours there was no measurable absorption of O_2. They inferred that, during the earlier oxidation stages at least, CO_2 and water are produced through the decomposition of the fat molecule itself without intervention of external oxygen. Although the early evolution of water and CO_2 in the autoxidation of fat has been observed before, the reactions concerned are obscure and appear worthy of closer investigation.

Greenbank & Weihe (1957) briefly described studies on the oxidative deterioration of butterfat at various temperatures with added anti-oxidants (butylated hydroxyanisole, nordihydroguaiaretic acid, and propyl gallate with and without citric acid). They commented that flavour changes appeared in butterfat before a peroxide value of 1·0 was reached, in contrast to other food fats which showed flavour stability up to much higher peroxide levels.

Smith, Frankel, Haab & Jack (1958) examined the effects of dissolved phospholipid and non-saponifiable fractions on the oxidative stability of butterfat at 80 °C. The known anti-oxidant properties of dissolved phospholipids were confirmed, milk- and vegetable-phospholipids being found equally effective in this respect. They reported also that acid-free unsaponifiable material from milk fat was anti-oxidant in itself and acted synergistically with phospholipids. Cholesterol appeared to have no effect on the rate of oxidation but milk-tocopherol was both anti-oxidant and exhibited synergism with phospholipids. Interestingly, in view of recent contrary findings, β-carotene was reported to have a slight pro-oxidant activity. In agreement with the results of Greenbank & Weihe (1957) oxidation in churned fat was detected organoleptically before the end of the induction period and at peroxide values less than 1·0.

(*b*) *Tests for fat oxidation.* The thiobarbituric acid (TBA) test for fat oxidation attracted considerable attention when first introduced about ten years ago, but interest in its application, to dairy products at least, appears to have waned. Difficulties in standardizing the test and lack of understanding of the basic nature of the reactions concerned appear to have hampered further development. Patton & Kurtz (1955), interested in the chemical basis of the test, noted that while the TBA reagent did not give the characteristic pink colour with freshly prepared α-, β-unsaturated aldehydes, it did so in the presence of copper or with oxidized samples (members of this class of compounds are believed to be responsible for oxidized flavours in milk and butter). McDowell (1955) made the interesting observation, which has yet to be explained, that the TBA test gave only a slight reaction with the fat from cold-stored

butter, whereas it gave a strong positive result with the serum of fresh and particularly with unsalted butter and an even stronger result with the serum of stored butter. Cerna, Masek & Vedlich (1957) described a modified Patton and Kurtz TBA test for butter using a citrate-HCl buffer for pH control and cobalt nitrate solutions for colour comparison as an alternative to photometric evaluation. The test was applied to the fat phase of the butter. The results of some tests on butter stored 6 months at -6 °C were presented; in general sweet-cream butters gave low TBA values ranging from 0 to 0·9 and acid butters higher values ranging up to 2·7.

A new test for oxidative rancidity in butterfat was described by Machulis (1957). Machulis found that steam distillates from oxidized butterfat exhibit strong ultraviolet absorption between 220 and 300 mμ with a maximum at 280 mμ and he developed a procedure for testing fat on this basis. He examined butterfat stored at room temperature over 6 months and found that the transmittancy of the distillates showed a slight but steady fall from the first week on and decreased sharply after 3 months, at which stage rancidity was evident. On the other hand, chemical and physical tests (specific gravity, refractive index, Kreis, aldehyde and peroxide tests, acid values, ketone reaction, oxidation, saponification and iodine numbers) showed no marked change until the sixth month. No indication was given of the nature of the materials in the distillates. Their water solubility suggests possible relationships with substances concerned in the TBA reaction. Further investigation and development of the Machulis test offers interesting possibilities.

(*c*) *Phospholipid oxidation.* Although trimethylamine from the decomposition of lecithin was thought for many years to be the cause of fishy flavour in butter, the accent in studies on the cold-storage deterioration of butter has in the past mainly been on glyceride oxidation. There is now a growing belief that oxidized flavour defects, in salted acid butters at least, are due to oxidation of the more highly unsaturated constituent phospholipid fatty acids and most of the results from recent work in this field support this theory. Lea (1957) discussed deteriorative reactions in foodstuffs involving phospholipids and lipoproteins and reviewed some of his own recent work on the isolation, fractionation and autoxidation of egg phospholipids. He pointed to the well-known oxidative instability of crude phospholipid preparations, the tendency to the inclusion of highly labile polyunsaturated acids in the phospholipid molecule and discussed the phenomenal oxidation rate (1 mole O_2 per mole of phospholipid per hour at 37 °C) of cephalin isolated chromatographically from egg yolk (Lea, 1955). Egg lecithin fractions oxidized much more slowly, in some cases at a rate only one hundredth that of the phosphatidyl-ethanolamine.

Swartling & Mattsson (1956), interested in the role of phospholipid in butter deterioration, studied the oxidation rate of methyl linoleate, with and without added milk phospholipids, at different acid reactions. Oxidation was followed by measuring O_2 uptake in a Warburg apparatus. Without phospholipid, oxygen uptake was faster at pH 6·2 than at pH 4·5. In the presence of phospholipid oxygen uptake was faster at the lower pH, the system showing the same relationship between pH and oxidation susceptibility as butter. This simple experiment points rather convincingly to an important and possibly decisive role for phospholipid in the oxidation of butter.

Koops & Pette (1956) also used a Warburg technique to study oxidation of milk phospholipid suspensions and obtained results consistent with those of Swartling

(1956); oxidation was markedly accelerated by lowering the pH, by the addition of copper and also by sodium chloride. A surprising observation was that copper bound to the phospholipids (by adding it to butter serum prior to solvent extraction) did not affect the rate of oxidation of the isolated phospholipids. Further experiments were described in which samples of fresh butterfat were mixed with oxidized phospholipid suspensions and immediately separated by centrifuging. The flavours absorbed in this way by the butterfat were described as trainy at first and becoming tallowy with increasing age of the phospholipid suspensions. Butterfat suspensions, similarly treated, did not yield any off-flavours.

Van Duin (1957) introduced a new element into the question of phospholipid reactivity and butter deterioration. Observing an unexpectedly large proportion of C14 to C18 aldehydes in the carbonyl compounds formed by autoxidation of isolated milk phospholipid at pH 4·6 (van Duin, 1958*a*, *b*), he suggested that these 'long' aldehydes were produced by hydrolytic separation from acetal phosphatides. In support of this theory he was able to isolate from 0·4 to 0·8 % of similar aldehydes by acid hydrolysis of unoxidized milk phospholipid (van Duin, 1958*c*). Saturated aldehydes of this chain-length would not be expected to have a strong flavour (van Duin stated they had a pleasant odour with a soapy flavour) and their role in butter deterioration has yet to be clarified.

(*d*) *Other forms of deterioration.* Keeney & Patton (1955*a*, 1956*a*, *b*) contributed to the understanding of flavour deterioration in butterfat by identifying δ-decalactone as the cause of a coconut-like flavour in this product. The defect appears in the early stages of storage of butterfat, dry whole milk and cream, sterilized and evaporated milks and appears to be unique to milk fat. The causative compound was isolated by chromatographic procedures from affected products and was identified on the basis of infra-red and paper chromatographic comparisons with authentic δ-decalactone. The origin of the coconut flavour defect is not clear, but it appears to arise through a fairly simple non-oxidative mechanism following heat treatment of milk fat. After investigation, 9-decenoic acid was eliminated as a possible precursor of the lactone in question (Keeney & Patton, 1955*b*).

Sampath, Anantakrishnan & Sen (1957) pointed out that although vanaspati was being fortified with synthetic vitamin A, little was known about the stability of the vitamin in this and other common edible fats in India. They found that vitamin A was most stable in cow ghee (15 % loss after 6 months at 37 °C) and least stable in sesame and groundnut oils (up to 100 % loss). Neither BHA nor ethyl gallate improved stability of vitamin A though fat peroxide formation was repressed. There was no difference between the stabilities in fat of added synthetic or natural vitamin A. Narayanan & Anantakrishnan (1957) suggested that surplus cow and buffalo colostral fat could be stored and used for feeding calves after reconstitution, and they examined the stability of vitamin A and carotene in these fats. They reported that both compounds were less stable in colostral fat than normal milk fat, the vitamin A content of cow colostral fat dropping to about half in some tests after 4 months at 37 °C. Keeping quality of the vitamin was better in buffalo colostral fats and was improved in both products by the addition of hydroquinone.

The thesis that high productivity in dairy cattle is accompanied by high globulin contents and increased susceptibility of milk fat to oxidation has been developed by

Pijanowski and co-workers (Pijanowski, Habaj & Hyziak, 1956, 1957; Pijanowski & Hyziak, 1956). They published supporting data showing the differences in globulin content between groups of high- and low-yielding Polish cows. The fat oxidation results, however, do not appear to be conclusive (susceptibility to oxidation was judged by peroxide development at 105–107 °C), the fat from the high-yielding cows being found most susceptible in the summer but least susceptible in the winter. A similar type of extreme heat treatment for testing the oxidation susceptibility of butterfat was used by Seitov (1957) who was interested in the form of deterioration of various fractions separated from butterfat by crystallization from ethyl alcohol. The fractions and the original butterfat were heated at 102 °C for 8 h and then stored at 5 °C for 12 months; oxidation was judged by peroxide formation. Deterioration in the more unsaturated fractions was oxidative in nature; in the fractions rich in volatile fatty acids hydrolysis and free acid development was pronounced. The original fat and the fractions from summer butter had better keeping quality than the corresponding fractions from winter butter; this was attributed to the higher content of carotenoids. The result, however, appears to be inconsistent with the generally reported inferior storage stability of summer, as opposed to winter butter in Europe.

Godel' (1956) described a greenish colour change occurring in stored butterfat, appearing first at the surface after 3–3½ months at −18 °C and then penetrating to the interior. In storage experiments with butterfat fractions precipitated from acetone the green colour appeared most readily in the more liquid fractions and at the lowest storage temperature (−18 °C). Spectroscopic examination of fresh fat in petrol showed an absorption maximum at 452 mμ similar to that of carotene. In affected fat the absorption curve broadened, with a secondary peak, into the ultra-violet region. The colour change was associated with changes in the carotene molecule presumably due to oxidation. The accelerating effect of lower temperatures was believed to be due to decreased stability of the carotene as the result of its increased concentration in the liquid fat fraction.

Weihe (1956) suggested a new approach to the problem of controlling fat oxidation in dairy products by partially hydrogenating butterfat. Experimental batches of butterfat were treated, using 0·5% Raney nickel catalyst under 1–4·4 atmospheres pressure and temperatures of 125 and 150 °C, and then submitted to a deodorizing process. It was reported that some degree of butter aroma remained with mild hydrogenation and that greatly improved storage performance was obtained. Rajan & Krukovsky (1957) studied the effects of molecular distillation at temperatures up to 215 °C and at atmospheric and reduced pressure on several fat constants and on oxidative stability. Though there were losses in vitamin A and carotenoids in the molecular distillation of tocopherol at 215 °C for 30 min there was no immediate effect on the susceptibility of the fat to oxidized flavours.

(4) *General*

(*a*) *Chromatography of butterfat.* Chromatographic methods have had a restricted application so far in the examination of milk fat and have been most successful in the separation of carotenoids and the fractionation of the saturated fatty acids. Most of the recent work with this technique has followed this trend. Garton & Lough (1957)

examined samples of butterfat by the Howard and Martin reversed phase technique which they extended to include the odd-numbered fatty acids with 9 to 19 C atoms. They reported good agreement between this method and conventional ester fractionation techniques for the higher saturated acids. Kuramoto, Jezeski & Holman (1957) showed that carrier displacement chromatography could be successfully used for separation of the common saturated fatty acids for butterfat. They found it necessary to use four chromatographic systems to cover the range from butyric to stearic acids. The samples were placed on the columns as soaps and acidified in place with sulphuric acid before being developed. Frankel, Smith & Jack (1958) attempted to separate some of the known constituents from milk fat by column chromatography with a view to studying their influence on the oxidative stability of the fat (Smith *et al.* 1958). Using silica columns and eluting with solvents of increasing polarity, fractionation of milk fat was not as complete as reported by previous workers for blood plasma lipids with similar techniques. Only cholesterol and phospholipids were separated, the tocopherols and carotenoids remaining with the triglycerides. Alumina and magnesia columns caused hydrolysis of the triglycerides but they were successfully used to separate cholesterol and fractionate carotenoids from the unsaponifiable matter. Studies on a column chromatographic method for determining simultaneously volatile and water-insoluble fatty acids (WIA) were described by van Dame (1957, 1958). On the basis of these results the method was adopted as 'First action' for butter and cream. (A review of the WIA test, its development and significance, was given by Hillig, 1956, 1958.)

Although gas chromatography was applied originally by James & Martin (1952) to the separation of fatty acids, few descriptions have been given of its use in the study of butterfat. Hawke (1957) used the technique to determine the total fatty acid composition of New Zealand butterfat. The volatile acids were separated with nitrogen gas and a mixture of a silicone, behenic acid and O-phosphoric acid as the liquid phase; the non-volatile acids were separated as the methyl esters with hydrogen and a vacuum grease liquid phase. The results obtained were in good agreement with those of other workers using conventional ester fractionation techniques. Hankinson & Harper (1957) briefly described a gas-liquid partition chromatographic method for the volatile fatty acids of milk using nitrogen gas, and silicone and stearic acid as the liquid phase on a kieselguhr support.

(*b*) *Other analytical methods.* Lagoni & Samhammer (1955) presented data showing the close connexion between iodine value and refractive index of butterfat and margarine fat. The relationship was found to be very close for butterfat with a correlation coefficient of 0·998 or 0·999, while the coefficient for margarine averaged 0·992. The slight spread of results with the margarine samples was attributed to the use of various types of fat.

Hammond & Eshelman (1957) gave a preliminary account of an investigation of Hilditch's and Kartha's methods of oxidizing glycerides to determine glyceride structure. By oxidizing model esters with and without acetic acid they found that Hilditch's method (Hilditch, 1956) caused some saponification of the triglycerides as originally reported by Kartha (1953). However, Kartha's method using acetic acid also gave side reactions with the introduction of new ester groups. The use of a number of alternative agents to acetic acid was unsuccessful.

According to Dushev (1956) no differences could be distinguished between the absorption spectra of cow and buffalo butters in the range 230–330 mμ. Schulz & Hetzel (1958) discussed the possibilities of spectrophotometric analysis in the examination of dairy products, particularly butterfat. They presented some spectrograms of butterfat taken with a Beckman DK 1 recording spectrophotometer over ranges from the near infra-red to ultra-violet and suggested that the technique could be used for characterizing butterfat and studying the effect of various treatments.

Jensen & Morgan (1957) found a little over 1 % of 1-monoglycerides in fat solvent extracted from rancid milk with Mojonnier reagents. The fat was partioned between heptane and 80 % ethanol and the 1-monoglyceride contents determined by periodic acid oxidation. Handwerk & Bird (1958) developed a method for the determination of unoxidized tocopherols in milk fat in order to follow their destruction during the oxidation of milk fat in storage. The method combines saponification in the presence of pyrogallol, chromatography on floridin treated with HCl and $SnCl_2$ and colour development in benzene-ethanol with $FeCl_3$ and bipyridyl.

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